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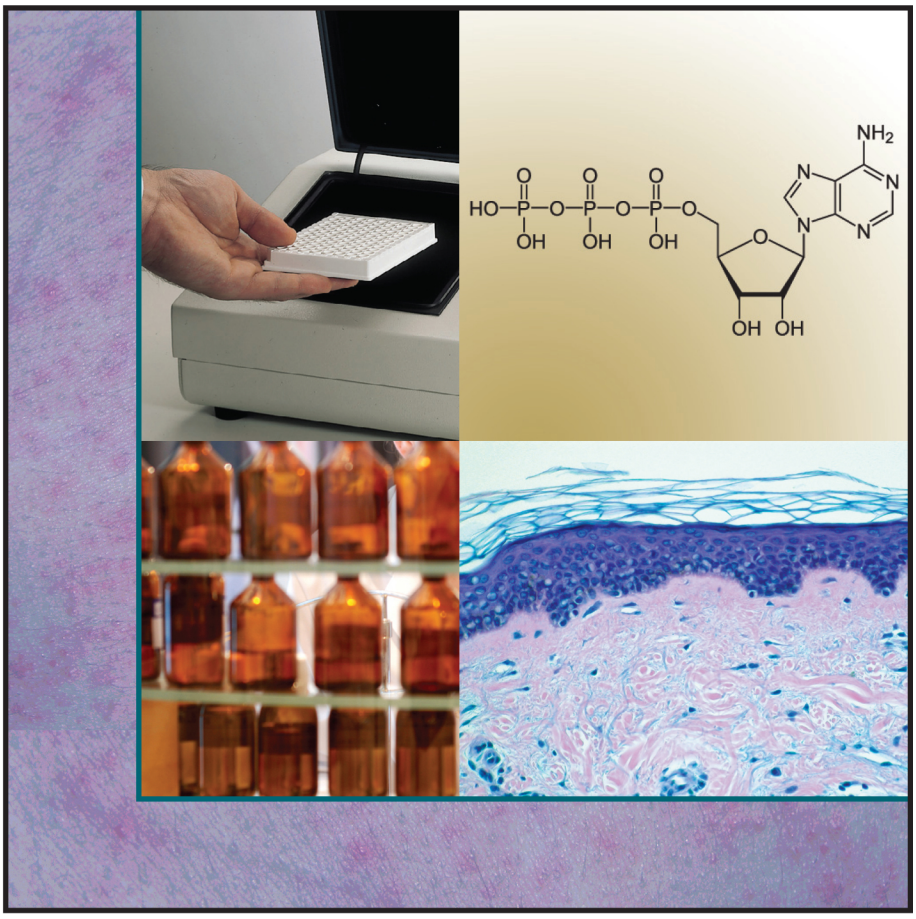


ICCVAM Test Method Evaluation Report on the Murine Local Lymph Node Assay: DA A Nonradioactive Alternative Test Method to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

National Institute of Environmental Health Sciences
National Institutes of Health
U.S. Public Health Service
Department of Health and Human Services

About the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

In 1997, the National Institute of Environmental Health Sciences (NIEHS), one of the National Institutes of Health, established ICCVAM to:

- Coordinate interagency technical reviews of new and revised toxicological test methods, including alternative test methods that reduce, refine, or replace the use of animals
- Coordinate cross-agency issues relating to validation, acceptance, and national and international harmonization of new, modified, and alternative toxicological test methods

On December 19, 2000, the ICCVAM Authorization Act (Public Law 106-545, 42 U.S.C. 285*l*-3) established ICCVAM as a permanent interagency committee of NIEHS under NICEATM.

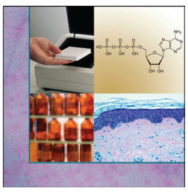
ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability. ICCVAM promotes the scientific validation and regulatory acceptance of toxicological test methods that more accurately assess the safety or hazards of chemicals and products and that reduce, refine (decrease or eliminate pain and distress), and/or replace animal use. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. More information about NICEATM and ICCVAM can be found on the NICEATM-ICCVAM website (http://iccvam.niehs.nih.gov) or obtained by contacting NICEATM (telephone: [919] 541-2384, e-mail: niceatm@niehs.nih.gov).

ICCVAM is an interagency committee with representatives from the following 15 U.S. Federal regulatory and research member agencies that require, use, generate, or disseminate toxicological information:*

- *Consumer Product Safety Commission*
- *Department of Agriculture*
- *Department of Defense*
- *Department of Energy*
- Department of Health and Human Services
  - Centers for Disease Control and Prevention
    - *Agency for Toxic Substances and Disease Registry*
    - *National Institute of Occupational Safety and Health*
  - *Food and Drug Administration*
  - National Institutes of Health
    - *Office of the Director*
    - *National Cancer Institute*
    - *National Institute of Environmental Health Sciences*
    - *National Library of Medicine*
- *Department of the Interior*
- Department of Labor
  - *Occupational Safety and Health Administration*
- *Department of Transportation*
- *Environmental Protection Agency*

*Italics indicate those agencies represented on ICCVAM, as specified in the ICCVAM Authorization Act.

The NICEATM-ICCVAM earth-and-sun graphic symbolizes the important role of new and alternative toxicological methods in protecting and advancing the health of people, animals, and our environment.



On the cover: This collage of pictures representing the murine local lymph node assay: DA (LLNA: DA) test method includes (clockwise from top left): a luminometer of the type used to measure ATP content in the LLNA: DA; a line drawing of the ATP molecule; a photomicrograph of a cross-section of human epidermis; brown bottles used for storage of light-sensitive chemicals or chemical mixtures. The lavender border below and to the left of the collage is a color-adjusted picture of a human skin rash. (Luminometer picture courtesy of Anselm Berthold/Berthold Detection Systems)

ICCVAM Test Method Evaluation Report on the Murine Local Lymph Node Assay: DA
A Nonradioactive Alternative Test Method to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products

Interagency Coordinating Committee on the Validation of Alternative Methods

National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

National Institute of Environmental Health Sciences
National Institutes of Health
U.S. Public Health Service
Department of Health and Human Services

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National Toxicology Program
P.O. Box 12233
Research Triangle Park, NC 27709

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List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
ACE	Acetone
AOO	Acetone: olive oil (4:1 by volume)
BRD	Background review document
BrdU	Bromodeoxyuridine
CASRN	Chemical Abstracts Service Registry Number
CI	Confidence interval
CMI	5-Chloro-2-methyl-4-isothiazolin-3-one
CPSC	U.S. Consumer Product Safety Commission
CV	Coefficient of variation
DMF	*N,N*-dimethylformamide
DMSO	Dimethyl sulfoxide
DNCB	2,4-Dinitrochlorobenzene
EC1.8	Estimated concentration needed to produce a stimulation index of 1.8
EC2.5	Estimated concentration needed to produce a stimulation index of 2.5
EC3	Estimated concentration needed to produce a stimulation index of 3.0
ECVAM	European Centre for the Validation of Alternative Methods
EGDMA	Ethylene glycol dimethacrylate
ELISA	Enzyme-linked immunosorbent assay
EPA	U.S. Environmental Protection Agency
ESAC	ECVAM Scientific Advisory Committee
FR	*Federal Register*
GP	Guinea pig
GPMT	Guinea pig maximization test
^{3}H	Tritiated
HCA	Hexyl cinnamic aldehyde
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ILS	Integrated Laboratory Systems
IWG	Immunotoxicity Working Group
JaCVAM	Japanese Center for the Validation of Alternative Methods
K_{ow}	Estimated log octanol-water partition coefficient
LLNA	Murine local lymph node assay
LLNA: DA	Murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content
LNC	Lymph node cells
Max.	Maximum
MBT	2-Mercaptobenzothiazole

MEK	Methyl ethyl ketone
NA	Not available
NC	Not calculated
Ni	Nickel
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences
No.	Number
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate buffered saline
rLLNA: DA	Reduced murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content
RLU	Relative luminescence units
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SD	Standard deviation
SEM	Standard error of the mean
SI	Stimulation index
SLS	Sodium lauryl sulfate
TCA	Trichloroacetic acid
TG	Test Guideline
U.K.	United Kingdom
U.S.	United States
U.S.C.	United States Code

Interagency Coordinating Committee on the Validation of Alternative Methods: Agency Representatives

Agency for Toxic Substances and Disease Registry
* Moiz Mumtaz, Ph.D.
Bruce Fowler, Ph.D.
Edward Murray, Ph.D.
Eric Sampson, Ph.D.

Consumer Product Safety Commission
* Marilyn L. Wind, Ph.D. (Chair)
+ Kristina Hatlelid, Ph.D.
Joanna Matheson, Ph.D.

Department of Agriculture
* Jodie Kulpa-Eddy, D.V.M. (Vice-Chair)
+ Elizabeth Goldentyer, D.V.M.

Department of Defense
* Robert E. Foster, Ph.D.
+ Patty Decot
Harry Salem, Ph.D.
Peter J. Schultheiss, D.V.M., DACLAM

Department of Energy
* Michael Kuperberg, Ph.D.
+ Marvin Stodolsky, Ph.D.

Department of the Interior
* Barnett A. Rattner, Ph.D.
+ Sarah Gerould, Ph.D. (to Feb. 2009)

Department of Transportation
* George Cushmac, Ph.D.
+ Steve Hwang, Ph.D.

Environmental Protection Agency
Office of Pesticide Programs
* John R. "Jack" Fowle III, Ph.D., DABT
+ Vicki Dellarco, Ph.D.
+ Tina Levine, Ph.D.
Deborah McCall
Christine Augustyniak, Ph.D. (*U.S. Coordinator, OECD Test Guidelines Program)*
Office of Pollution Prevention and Toxics
Jerry Smrchek, Ph.D. (*U.S. Coordinator, OECD Test Guidelines Program,* to July 2009*)*
Office of Research and Development
Suzanne McMaster, Ph.D. (to Dec. 2008)
Julian Preston, Ph.D. (to July 2009)
Stephanie Padilla, Ph.D. (to July 2009)
Office of Science Coordination and Policy
Karen Hamernik, Ph.D. (to July 2009)

Food and Drug Administration
Office of the Commissioner
* Suzanne Fitzpatrick, Ph.D., DABT
Center for Biologics Evaluation and Research
Richard McFarland, Ph.D., M.D.
Ying Huang, Ph.D.
Center for Devices and Radiological Health
Melvin E. Stratmeyer, Ph.D.
Vasant G. Malshet, Ph.D., DABT
Center for Drug Evaluation and Research
+ Abigail C. Jacobs, Ph.D.
Paul C. Brown, Ph.D.
Center for Food Safety and Applied Nutrition
David G. Hattan, Ph.D.
Robert L. Bronaugh, Ph.D.
Center for Veterinary Medicine
Devaraya Jagannath, Ph.D.
M. Cecilia Aguila, D.V.M.
National Center for Toxicological Research
Paul Howard, Ph.D.
Donna Mendrick, Ph.D.
William T. Allaben, Ph.D. (to Jan. 2009)
Office of Regulatory Affairs
Lawrence D'Hoostelaere, Ph.D.

National Cancer Institute
* T. Kevin Howcroft, Ph.D.
Chand Khanna, D.V.M., Ph.D.
Alan Poland, M.D. (to Oct. 2008)

National Institute of Environmental Health Sciences
* William S. Stokes, D.V.M., DACLAM
+ Raymond R. Tice, Ph.D.
Rajendra S. Chhabra, Ph.D., DABT
Jerrold J. Heindel, Ph.D.

National Institute for Occupational Safety and Health
* Paul Nicolaysen, V.M.D.
+ K. Murali Rao, M.D., Ph.D.

National Institutes of Health
* Margaret D. Snyder, Ph.D.

National Library of Medicine
* Pertti (Bert) Hakkinen, Ph.D.
+ Jeanne Goshorn, M.S.

Occupational Safety and Health Administration
* Surender Ahir, Ph.D.

* Principal agency representative
+ Alternate principal agency representative

Acknowledgements

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Immunotoxicity Working Group (IWG)

U.S. Consumer Product Safety Commission
Joanna Matheson, Ph.D. (IWG Co-chair)
Marilyn L. Wind, Ph.D.

U.S. Environmental Protection Agency
Office of Pesticide Programs
Jonathan Chen, Ph.D.
Masih Hashim, D.V.M., Ph.D.
Marianne Lewis
Deborah McCall
Timothy McMahon, Ph.D.
John Redden, M.S.
Jenny Tao, Ph.D.
Office of Pollution Prevention and Toxics
Elizabeth Margosches, Ph.D.
Ronald Ward, Ph.D.
Office of Research and Development
Marsha Ward, Ph.D.
Office of Science Coordination and Policy
Karen Hamernik, Ph.D.

U.S. Food and Drug Administration
Center for Devices and Radiological Health
Vasant G. Malshet, Ph.D., DABT
Jeffrey Toy, Ph.D.
Center for Drug Evaluation and Research
Ruth Barratt, Ph.D., D.V.M.
Paul C. Brown, Ph.D.
Abigail C. Jacobs, Ph.D. (IWG Co-chair)
Jiaqin Yao, Ph.D.
Office of Science and Health Coordination
Suzanne Fitzpatrick, Ph.D., DABT

National Institute of Environmental Health Sciences
Dori Germolec, Ph.D.
William S. Stokes, D.V.M., DACLAM

National Institute for Occupational Safety and Health
B. Jean Meade, D.V.M., Ph.D.

National Library of Medicine
Pertti Hakkinen, Ph.D.

European Centre for the Validation of Alternative Methods – Liaison
Silvia Casati, Ph.D.
Alexandre Angers, Ph.D.

Japanese Center for the Validation of Alternative Methods – Liaison
Hajime Kojima, Ph.D.

Murine Local Lymph Node Assay
Independent Scientific Peer Review Panel
(March 4-6, 2008 and April 28-29, 2009)

Michael Luster, Ph.D. (Panel Chair)
Senior Consultant to the National Institute for Occupational Safety and Health
Health Effects Laboratory
Morgantown, WV

Nathalie Alépée, Ph.D.
Scientific Coordinator on Alternatives Methods in Life Science
L'Oréal Research and Development
Aulnay sous Bois, France

Anne Marie Api, Ph.D.
Vice President, Human Health Sciences
Research Institute for Fragrance Materials
Woodcliff Lake, NJ

Nancy Flournoy, M.S., Ph.D.
Professor and Chair
Department of Mathematics and Statistics
University of Missouri – Columbia
Columbia, MO

Thomas Gebel, Ph.D.[1]
Regulatory Toxicologist
Federal Institute for Occupational Safety & Health
Dortmund, Germany

Kim Headrick, B.Admin., B.Sc.[1]
International Harmonization and Senior Policy Advisor
Policy and Programme Service Office
Health Canada
Ottawa, Ontario, Canada

Dagmar Jírová, M.D., Ph.D.
Toxicologist, Research Manager
Head of Reference Center for Cosmetics and Reference Laboratory for Experimental Immunotoxicology
National Institute of Public Health
Prague, Czech Republic

David Lovell, Ph.D., FIBiol, CStat, CBiol
Reader in Medical Statistics
Postgraduate Medical School
University of Surrey
Guildford, Surrey, U.K.

Howard Maibach, M.D.
Professor, Department of Dermatology
University of California – San Francisco
San Francisco, CA

James McDougal, Ph.D.[1]
Professor and Director of Toxicology Research
Department of Pharmacology and Toxicology
Boonshoft School of Medicine
Wright State University
Dayton, OH

Michael Olson, Ph.D., A.T.S.
Director of Occupational Toxicology
Corporate Environment, Health and Safety
GlaxoSmithKline
Research Triangle Park, NC

Raymond Pieters, Ph.D.[2]
Associate Professor
Immunotoxicology Group Leader
Institute for Risk Assessment Sciences
Utrecht University
Utrecht, The Netherlands

Jean Regal, Ph.D.
Professor, Department of Pharmacology
University of Minnesota Medical School
Duluth, MN

Jonathan Richmond, MB ChB, FRCSEd[3]
Head, Animals Scientific Procedures Division
Home Office
London, U.K.

Peter Theran, V.M.D.
Consultant
Massachusetts Society for the Prevention of Cruelty to Animals
Novato, CA

Stephen Ullrich, Ph.D.
Professor of Immunology
Graduate School of Biomedical Sciences
University of Texas
M.D. Anderson Cancer Center – Houston
Houston, TX

Michael Woolhiser, Ph.D.
Science and Technology Leader
Toxicology and Environmental Research and Consulting
The Dow Chemical Company
Midland, MI

Takahiko Yoshida, M.D., Ph.D.
Professor, Department of Health Science
Asahikawa Medical College
Hokkaido, Japan

[1] Drs. Gebel and McDougal and Ms. Headrick were unable to attend the public meeting on April 28-29, 2009, and did not participate in the review.

[2] Dr. Pieters was unable to attend the public meeting on April 28-29, 2009. However, he was involved in the peer review of the documents and concurred with the conclusions and recommendations included in the *Independent Scientific Peer Review Panel Report – Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products.*

[3] Dr. Richmond was unable to attend the public meeting on March 4-6, 2008. However, he was involved in the peer review of the documents and concurred with the conclusions and recommendations included in the *Independent Scientific Peer Review Panel Report – Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products.*

National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

National Institute of Environmental Health Sciences

William Stokes, D.V.M., DACLAM
Director; Project Officer

Deborah McCarley
Special Assistant; Assistant Project Officer

NICEATM Support Contract Staff (Integrated Laboratory Systems [ILS], Inc.)

David Allen, Ph.D.
Thomas Burns, M.S.
Linda Litchfield
Steven Morefield, M.D.
Michael Paris
Eleni Salicru, Ph.D.
Catherine Sprankle
Frank Stack
Judy Strickland, Ph.D., DABT
Linda Wilson

Statistical Consultant for ILS, Inc.

Joseph Haseman, Ph.D.

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Kenji Idehara, Ph.D.
Daicel Chemical Industries, Ltd.
Hyogo, Japan

Takashi Omori, Ph.D.
Kyoto University School of Public Health
Kyoto, Japan

Preface

Allergic contact dermatitis (ACD) is an adverse health effect that frequently develops in workers and consumers exposed to skin sensitizing chemicals and products. ACD results in lost workdays[1] and can significantly diminish quality of life (Hutchings et al. 2001; Skoet et al. 2003). To minimize the occurrence of ACD, regulatory authorities require testing to identify substances that may cause skin sensitization. Sensitizing substances must be labeled with a description of the potential hazard and the precautions necessary to avoid development of ACD.

Skin sensitization testing has typically required the use of guinea pigs (Buehler 1965; Magnusson and Kligman 1970). However, in 1998, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) evaluated and recommended an alternative test method known as the murine (mouse) local lymph node assay ("traditional LLNA").[2] The traditional LLNA provides several advantages compared to guinea pig test methods, including elimination of potential pain and distress, use of fewer animals, less time to perform, and availability of dose-response information. Based on the validation database and performance, ICCVAM recommended the LLNA as an alternative test method for assessing the skin sensitization potential of most types of substances (ICCVAM 1999). United States and international regulatory agencies subsequently accepted the traditional LLNA as a valid alternative test method for ACD testing.

In 2007, the U.S. Consumer Product Safety Commission (CPSC) requested that ICCVAM evaluate several modifications of the traditional LLNA, including a nonradioactive version of the LLNA developed by Dr. Kenji Idehara at Daicel Chemical Industries, Ltd. in Hyogo, Japan. This version (referred to as the "LLNA: DA") measures increases in ATP content instead of using a radioactive marker to measure lymphocyte proliferation. The validation studies were completed in coordination with the Japanese Center for the Validation of Alternative Methods (JaCVAM) at the National Institute of Health Sciences. ICCVAM assigned this activity a high priority after considering comments from the public and ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods (SACATM). As part of their ongoing collaboration with ICCVAM, scientists from the European Centre for Validation of Alternative Methods (ECVAM) and JaCVAM served as liaisons to the ICCVAM Immunotoxicity Working Group (IWG). A detailed timeline of the LLNA: DA evaluation is included with this report.

This Test Method Evaluation Report provides ICCVAM's recommendations regarding the LLNA: DA for assessing the ACD hazard potential of chemicals and products. Since the LLNA: DA does not require the use of a radioactive marker, it can be used by laboratories that currently cannot use the traditional LLNA because they do not have a license for using radioisotopes and in countries that severely limit or discourage the use of radioactive materials required by the traditional LLNA. The report also summarizes the validation status of the LLNA: DA and provides the ICCVAM-recommended LLNA: DA test method protocol.

Following independent scientific peer reviews in 2008 and 2009, ICCVAM submitted a proposed draft Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) for the LLNA: DA that was circulated in July 2009 to the 30 OECD member countries for review and comment. The U.S. CPSC and NICEATM-ICCVAM hosted an OECD Expert Consultation meeting on October 20-22, 2009, to evaluate the comments. A revised TG was distributed to the 30 OECD member countries in December 2009 for comment and then the final draft was forwarded to the

[1] http://www.blf.gov/IIF

[2] The "traditional LLNA" refers to the ICCVAM-recommended LLNA test method protocol, which measures lymphocyte proliferation based on incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine into the cells of the draining auricular lymph nodes (ICCVAM 1999; Dean et al. 2001).

OECD Working Group of National Co-ordinators of the Test Guidelines Programme, which was approved as TG 442A at their March 23-25, 2010 meeting.

ICCVAM solicited and considered public comments and stakeholder involvement throughout the LLNA: DA evaluation process. ICCVAM considered the SACATM comments, the conclusions of the Panel and the OECD Expert Consultation, and all public comments before finalizing the ICCVAM test method recommendations for the LLNA: DA. The recommendations and the Background Review Document, which is provided as an appendix to this report, are incorporated in this ICCVAM Test Method Evaluation Report. As required by the ICCVAM Authorization Act (2000; Public Law 106-545, 42 United States Code 285*l*-3), ICCVAM will forward its recommendations to U.S. Federal agencies for consideration. Federal agencies must respond to ICCVAM within 180 days after receiving the ICCVAM test method recommendations. ICCVAM recommendations are available to the public on the NICEATM-ICCVAM website[3] and agency responses will also be made available on the website as they are received.

We gratefully acknowledge the many individuals who contributed to the preparation, review, and revision of this report. We especially recognize the Panel members for their thoughtful evaluations and generous contributions of time and effort. Special thanks are extended to Dr. Michael Luster for serving as the Panel Chair and to Dr. Michael Woolhiser, Dr. Michael Olson, Dr. Stephen Ullrich, and Kim Headrick for their service as Evaluation Group Chairs. We thank the IWG for assuring a meaningful and comprehensive review. We especially thank Dr. Joanna Matheson (CPSC) and Dr. Abigail Jacobs (U.S. Food and Drug Administration Center for Drug Evaluation and Research) for serving as Co-chairs of the IWG. We also acknowledge Integrated Laboratory Systems, Inc., the NICEATM support contractor, for providing excellent scientific and operational support, including Dr. David Allen, Thomas Burns, Michael Paris, Dr. Eleni Salicru, Frank Stack, and Dr. Judy Strickland. Finally, we thank Dr. Silvia Casati and Dr. Hajime Kojima, the IWG liaisons from ECVAM and JaCVAM, respectively, for their participation and contributions.

This comprehensive ICCVAM evaluation of the LLNA: DA should facilitate regulatory agency decisions on the acceptability of the method. Use of the method by industry can be expected to significantly reduce and refine animal use required for ACD testing while continuing to support the protection of human health.

Marilyn Wind, Ph.D.
Deputy Associate Executive Director
Directorate for Health Sciences
U.S. Consumer Product Safety Commission
Chair, ICCVAM

RADM William S. Stokes, D.V.M., DACLAM
Rear Admiral/Assistant Surgeon General, U.S. Public Health Service
Director, NICEATM
Executive Director, ICCVAM

[3] http://iccvam.niehs.nih.gov/methods/immunotox/llna-DA/TMER.htm

Executive Summary

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recently evaluated the validation status of a nonradioactive version of the murine local lymph node assay (LLNA) called the LLNA modified by Daicel Chemical Industries, Ltd., based on ATP content (LLNA: DA). The LLNA is used to identify chemicals and products that may cause allergic contact dermatitis (ACD), an allergic skin reaction characterized by redness, swelling, and itching. The LLNA: DA measures increases in ATP content by luciferin-luciferase assay as an indicator of increases in lymphocyte cell number while the traditional LLNA uses ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine uptake to measure lymphocyte proliferation.[4] This Test Method Evaluation Report provides ICCVAM's recommendations regarding the usefulness and limitations of the LLNA: DA as a variation of the traditional LLNA. The report includes the ICCVAM-recommended LLNA: DA test method protocol, the final LLNA: DA background review document (BRD) describing the validation status of the test method, and recommendations for future studies and performance standards.

Following nomination of the LLNA: DA by the U.S. Consumer Product Safety Commission (CPSC), the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), ICCVAM, and the ICCVAM Immunotoxicity Working Group prepared an initial draft BRD and draft test method recommendations. The drafts were provided to an independent international scientific peer review panel (Panel) and the public for comment. The Panel met twice in public session to review the initial and revised draft BRDs and draft ICCVAM recommendations. The initial draft BRD evaluated data for 29 substances. The Panel initially met in public session on March 4-6, 2008, to discuss its peer review of the ICCVAM draft BRD and to provide conclusions and recommendations regarding the validation status of the LLNA: DA test method. The Panel also reviewed how well the information in the draft BRD supported ICCVAM's draft test method recommendations. The Panel concluded that definitive test method recommendations could not be made until a detailed protocol and individual animal data were obtained and an evaluation of interlaboratory reproducibility was conducted.

NICEATM revised the draft BRD with additional information and data. The revised draft BRD evaluated data for 44 substances. The Panel reconvened in public session on April 28-29, 2009, to review the ICCVAM revised draft BRD and to finalize its conclusions and recommendations on the current validation status of the LLNA: DA test method.

Based on the revised draft ICCVAM recommendations and Panel reports, NICEATM submitted a proposed draft Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) for the LLNA: DA. The draft TG was circulated in July 2009 to the 30 OECD member countries for review and comment. The U.S. CPSC and NICEATM-ICCVAM hosted an OECD Expert Consultation meeting on October 20-22, 2009, to evaluate the comments. The expert group reviewed the draft OECD TG for the LLNA: DA and proposed responses to comments from member countries. A revised TG was distributed to the 30 OECD member countries in December 2009 for comment and then the final draft was forwarded to the OECD Working Group of National Co-ordinators of the Test Guidelines Programme, which approved the LLNA: DA as TG 442A at their March 23-25, 2010 meeting.

In finalizing this Test Method Evaluation Report and the BRD, which is included as an appendix, ICCVAM considered (1) the conclusions and recommendations of the Panel and the OECD Expert Consultation, (2) comments from ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), and (3) public comments.

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[4] *Traditional LLNA* refers to the ICCVAM-recommended LLNA protocol, which measures lymphocyte proliferation based on incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine into the cells of the draining auricular lymph nodes (ICCVAM 1999; Dean et al. 2001).

ICCVAM Recommendations: Test Method Usefulness and Limitations

ICCVAM concludes that the accuracy and reliability of the LLNA: DA support use of the test method to identify substances as potential skin sensitizers and nonsensitizers. For the validation database of 44 substances, the LLNA: DA correctly identified all 32 LLNA sensitizers (0% [0/32] false negatives), and nine of the 12 LLNA nonsensitizers (25% [3/12] false positives).[5] ICCVAM recommends that a stimulation index (SI) ≥ 1.8 be used as the decision criterion to identify substances as potential sensitizers. ICCVAM bases this recommendation on the fact that no false negatives, relative to the traditional LLNA, result with the current validation database when an SI ≥ 1.8 is used.

A limitation of the LLNA: DA is the potential for false positive results when borderline positive responses between an SI of 1.8 and 2.5 are obtained. Further, the use of the LLNA: DA might not be appropriate for testing substances that affect ATP levels (e.g., substances that function as ATP inhibitors) or those that affect the accurate measurement of intracellular ATP (e.g., presence of ATP degrading enzymes, presence of extracellular ATP in the lymph node).

ICCVAM Recommendations: Test Method Protocol

The ICCVAM-recommended LLNA: DA test method protocol, which is based on the protocol developed by Yamashita et al. (2005) and Idehara et al. (2008), incorporates all aspects of the ICCVAM-recommended traditional LLNA test method protocol except for those procedures unique to the conduct of the LLNA: DA. In testing situations that do not require dose-response information, or negative results are anticipated, the LLNA: DA should be considered for use as a reduced test method protocol. The reduced LLNA: DA tests only the high dose, thus further reducing animal use.

ICCVAM Recommendations: Future Studies

To further characterize the LLNA: DA test method, ICCVAM recommends that efforts be made to identify additional human data and human experience for test substances. These data may be used to further assess the usefulness and limitations of this and other versions of the LLNA for identifying human sensitizing substances. Such efforts might include postmarketing surveillance of consumers for allergic reactions and occupational surveillance of potentially exposed workers. Additional nonsensitizing skin irritants should be tested to determine the impact of such substances on the false positive rate of the LLNA: DA.

ICCVAM also recommends that efforts be made to further characterize the sensitization potential of borderline positive substances that produce SI values between 1.8 and 2.5 to determine if such results might be false positives. This could include (1) evaluations of peptide reactivity; (2) determination of molecular weight; (3) identification of results from related chemicals; (4) human studies where ethically and scientifically justified; and (5) review of occupational exposures, postmarketing experience or monitoring, and/or *in vitro* testing data. All decision criteria should be reassessed as additional discriminators and data become available.

ICCVAM Recommendations: Performance Standards

The ICCVAM-recommended performance standards for the traditional LLNA (ICCVAM 2009a) apply to the LLNA: DA because the test method is functionally and mechanistically similar to the traditional LLNA. Therefore, ICCVAM recommends that the ICCVAM-recommended performance standards for the traditional LLNA be used to evaluate any future modifications of the LLNA: DA.

Validation Status of the LLNA: DA

The mechanistic basis of the LLNA: DA is identical to that of the traditional LLNA. The traditional LLNA measures the lymphocyte proliferation in the draining lymph nodes for the skin area where the test article is applied. In the traditional LLNA, lymphocyte proliferation three-fold or more higher

[5] These results used the most prevalent outcome for substances that were tested multiple times.

than the vehicle control is considered a positive response indicative of a skin sensitizing substance. The LLNA: DA assesses cell proliferation by measuring increases in ATP content in the draining auricular lymph nodes as an indicator of cell number. The LLNA: DA also differs from the traditional LLNA in the test substance treatment and sampling schedule. In addition, the LLNA: DA includes pretreatment of the application site with an aqueous solution of 1% sodium lauryl sulfate (SLS).

The accuracy of the LLNA: DA was compared to that of the traditional LLNA. Optimal LLNA: DA performance was achieved using SI ≥ 1.8 to classify sensitizers versus nonsensitizers. Compared to the traditional LLNA, accuracy was 93% (41/44), with a false positive rate of 25% (3/12) and a false negative rate of 0% (0/32). The three false positive substances using SI ≥ 1.8 produced SI values between 1.8 and 2.5 in the LLNA: DA. Therefore, other available information, such as dose-response, evidence of systemic toxicity or excessive local irritation, and where appropriate, statistical significance together with SI values should be considered to confirm that such borderline positive results are potential skin sensitizers. Consideration should also be given to various properties of the test substance, including whether it is structurally similar to known skin sensitizers.

An evaluation to determine the robustness of the optimum SI ≥ 1.8 decision criterion indicated that the SI was quite stable. Taking different samples of the data as training and validation sets had relatively little impact on the cutoff SI criterion or on the resulting number of false or false negative results.

ICCVAM concludes that the reproducibility of the LLNA: DA supports the use of the method to identify substances as potential skin sensitizers and nonsensitizers. The validation database supported an assessment of both intra- and interlaboratory reproducibility. A two-phased study was conducted to assess interlaboratory reproducibility.

Intralaboratory reproducibility was assessed using a coefficient of variation (CV) analysis of EC3 (estimated concentration needed to produce an SI of 3.0) and EC1.8 values (estimated concentration needed to produce an SI of 1.8) for isoeugenol and eugenol. (Each substance was tested in three different experiments.) The mean EC3 value for isoeugenol was 2.74% ± 0.58%, with a corresponding CV of 21%. Eugenol had an EC3 of 5.06% ± 0.55% and a CV of 11%. The mean EC1.8 value and corresponding CV for isoeugenol and eugenol were 0.87% ± 0.31% (36% CV) and 3.38% ± 0.79% (23% CV), respectively.

Both phases of an interlaboratory validation study included qualitative analyses of LLNA: DA reproducibility. An SI ≥ 1.8 was used as the threshold to distinguish sensitizers from nonsensitizers. In the first phase, 12 substances (nine sensitizers and three nonsensitizers based on traditional LLNA test results) were tested in either three or 10 laboratories. There was 100% agreement among the laboratories for 10 substances (seven sensitizers and three nonsensitizers based on traditional LLNA results). There was 67% (2/3) agreement among the tests for the remaining two traditional LLNA sensitizers. Interlaboratory CV values for the EC1.8 values of the nine sensitizers ranged from 15% to 140%.

The second phase included five substances (four sensitizers and one nonsensitizer based on traditional LLNA test results) tested in either four or seven laboratories. There was 100% agreement among the laboratories for four substances (three sensitizers and one nonsensitizer based on traditional LLNA results). There was 75% (3/4) agreement among the tests for the remaining traditional LLNA sensitizer. Interlaboratory CV values for the EC1.8 values of the four traditional LLNA sensitizers ranged from 14% to 93%.

Reproducibility of results for the 14 substances (10 traditional LLNA sensitizers and four traditional LLNA nonsensitizers) that had three to 18 test results, regardless of whether the tests were performed in one laboratory or multiple laboratories, was assessed with respect to SI category. When the SI ≥ 1.8 decision criterion was used to classify sensitizers versus nonsensitizers the SI results for 80%

(8/10) of the sensitizers (based on traditional LLNA results) were 100% concordant (i.e., all tests for that substance yielded maximum SI ≥ 1.8) in the LLNA: DA for three to 18 tests. The SI results for 75% (3/4) of the nonsensitizers (based on traditional LLNA results) were 100% concordant in the LLNA: DA (i.e., all tests for that substance yielded SI < 1.8) for four to 11 tests. The other nonsensitizer had 91% concordance (10/11). This test for the nonsensitizer yielded SI values between 1.8 and 2.5, the narrow region in which false positive results occurred.

ICCVAM Consideration of Independent Peer Review Panel Report and Other Comments

The ICCVAM evaluation process incorporates a high level of scientific peer review and transparency. The evaluation process for the LLNA: DA included two public review meetings by an independent scientific peer review panel, multiple opportunities for public comments, consideration of reports from an OECD Consultation, and comments from the SACATM. ICCVAM and the Immunotoxicity Working Group considered the Panel report, conclusions of the OECD Expert Consultation, the SACATM comments, and all public comments before finalizing the ICCVAM Test Method Evaluation Report and final BRD for the LLNA: DA.

1.0 Introduction

The murine local lymph node assay (traditional LLNA)[1] is an alternative skin-sensitization test method that requires fewer animals and less time than currently accepted guinea pig tests (e.g., the guinea pig maximization test [GPMT] and the Buehler test). It also avoids animal discomfort that can occur in the guinea pig tests when substances cause allergic contact dermatitis (ACD). The LLNA measures cell proliferation in the draining auricular lymph nodes of the mouse by analyzing incorporation of a radioactive marker into newly synthesized DNA. The LLNA was the first alternative test method evaluated and recommended by the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). International regulatory authorities have now recognized the traditional LLNA as an acceptable alternative to guinea pig tests for most testing situations.

The LLNA modified by Daicel Chemical Industries, Ltd., based on ATP content (referred to hereafter as the "LLNA: DA") was one of several modified versions of the LLNA nominated by the U.S. Consumer Product Safety Commission (CPSC) for evaluation by ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM).[2] It is a nonradioactive version of the LLNA that assesses cell proliferation by detecting increases in ATP content as an indicator of cell number at the end of cell proliferation rather than by quantifying the incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine. The increase in ATP content in lymph nodes from test animals compared to vehicle control animals is then quantified using a luciferin-luciferase assay. The LLNA: DA can reduce the use of animals for skin sensitization testing when it is used in place of guinea pig tests in countries that severely limit or discourage the use of radioactive materials that are required by the traditional LLNA.

In accordance with the ICCVAM Authorization Act of 2000 (Public Law 106-545, 42 United States Code 285*l*-3), ICCVAM coordinates the technical evaluations of new, revised, and alternative test methods with regulatory applicability. After considering comments from the public and ICCVAM's advisory committee, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), ICCVAM members unanimously agreed that the LLNA: DA should have a high priority for evaluation. A detailed timeline of the LLNA: DA evaluation is provided in **Appendix A**. The ICCVAM-recommended LLNA: DA test method protocol and the final LLNA: DA background review document (BRD) are provided in **Appendices B** and **C**, respectively.

The ICCVAM Immunotoxicity Working Group (IWG) was established to work with NICEATM to evaluate the LLNA: DA and other test methods and applications. The European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM) designated liaison members to the IWG.

To facilitate peer review of the LLNA: DA test method, the IWG and NICEATM prepared a comprehensive draft BRD that provided information and data from validation studies and the scientific literature. A May 17, 2007, *Federal Register* (FR) notice (72 FR 27815)[3] requested data and information on these test methods and nominations of individuals to serve on an international independent scientific peer review panel (Panel). The request was also disseminated via the ICCVAM electronic mailing list and through direct requests to over 100 stakeholders. In response to this request, one individual submitted LLNA: DA data and three individuals or organizations nominated members to the Panel (see **Section 4.0**).

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[1] The "traditional LLNA" refers to the ICCVAM-recommended LLNA test method protocol, which measures lymphocyte proliferation based on incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine into the cells of the draining auricular lymph nodes (ICCVAM 1999; Dean et al. 2001).

[2] Available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf

[3] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf

In the initial draft BRD, ICCVAM examined data for 29 substances with adequate traditional LLNA data (19 sensitizers and 10 nonsensitizers, as classified by the traditional LLNA) that were tested in a single laboratory (Idehara et al. 2008). On January 8, 2008, ICCVAM announced the availability of the draft BRD to the public and a public Panel meeting to review the validation status of the LLNA: DA (and other LLNA-related activities) (73 FR 1360).[4] All of the information provided to the Panel, including the ICCVAM draft BRD, draft test method recommendations, and all public comments received prior to the Panel meeting, were made publicly available via the NICEATM-ICCVAM website.[5]

The first Panel meeting was a public session held on March 4-6, 2008, to review the validation status of the LLNA: DA and the completeness of the ICCVAM draft BRD (see **Appendix D**). The Panel evaluated (1) the extent to which the draft BRD addressed established validation and acceptance criteria and (2) the extent to which the draft BRD supported ICCVAM's draft proposed test method uses, recommended test method protocol, draft test method performance standards, and proposed future studies. Interested stakeholders from the public were provided opportunities to comment at the Panel meeting. The Panel considered these comments as well as those submitted prior to the meeting before concluding their deliberations. The Panel agreed with the draft ICCVAM recommendations that the LLNA: DA may be useful for identifying substances as potential skin sensitizers and nonsensitizers, but that more information and data were needed before definitive conclusions on the usefulness and limitations of the LLNA: DA could be made. The Panel noted that the following information was needed before definitive recommendations could be made: (1) a detailed test method protocol; (2) individual animal data for the validation database; and (3) an evaluation of interlaboratory reproducibility. On May 20, 2008, ICCVAM posted a report of the Panel's recommendations[6] (see **Appendix D**) on the NICEATM-ICCVAM website for public review and comment (announced in 73 FR 29136).[7]

ICCVAM provided SACATM with the draft BRD and draft test method recommendations, the Panel report, and all public comments for discussion at their meeting on June 18-19, 2008, where public stakeholders were given another opportunity to comment.

NICEATM subsequently obtained a detailed test method protocol and additional data and revised the draft BRD to include this new information. The revised draft BRD included an accuracy evaluation for the expanded database of individual animal results for 44 substances with adequate traditional LLNA data (32 sensitizers and 12 nonsensitizers, as classified by the traditional LLNA) as well as an evaluation of interlaboratory reproducibility. Based on the analyses included in the revised draft BRD, ICCVAM prepared revised draft test method recommendations for proposed test method uses and limitations, recommended test method protocol, test method performance standards, and future studies for the LLNA: DA.

On November 4, 2008, JaCVAM released a statement that at a meeting concerning the LLNA: DA at the National Institute of Health Sciences, Tokyo, Japan, on August 28, 2008, the noncommissioned members of the JaCVAM Regulatory Acceptance Board unanimously endorsed the following statement (see **Appendix E**): "Following the review of the results of the Ministry of Health, Labour and Welfare-funded validation study of the LLNA: DA coordinated by the Japanese Society for Alternative to Animal Experimentation, it is concluded that the LLNA: DA can be used for distinguishing between sensitizer and nonsensitizer chemicals within the context of the Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 429 on skin sensitization: LLNA."

[4] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_25553.pdf
[5] Available at http://iccvam.niehs.nih.gov
[6] Available at http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[7] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E8-11195.pdf

ICCVAM released the revised draft documents to the public for comment on February 27, 2009, and announced a second meeting of the Panel (74 FR 8974).[8] The Panel reconvened on April 27-28, 2009, to reassess the validation status of the LLNA: DA (see **Appendix D**). The Panel also reviewed the completeness of the revised draft ICCVAM BRD and the extent to which the information therein supported the revised draft ICCVAM test method recommendations. On June 1, 2009, ICCVAM posted the second report of the Panel's recommendations[9] (see **Appendix D**) on the NICEATM-ICCVAM website for public review and comment (announced in 74 FR 26242).[10]

ICCVAM provided SACATM with the revised draft BRD, the second Panel report, and all public comments for discussion at their meeting on June 25-26, 2009, where public stakeholders were given another opportunity to comment.

Based on the revised draft ICCVAM recommendations, NICEATM submitted a proposed draft OECD TG for the LLNA: DA that was circulated in July 2009 to the 30 OECD member countries for review and comment via their National Co-ordinators, who distributed the draft TG to interested stakeholders. An OECD Expert Consultation Meeting was held on October 20-22, 2009, to evaluate the comments. Scientists from the National Institute of Environmental Health Sciences (NIEHS), the Environmental Protection Agency, the Food and Drug Administration, and the CPSC, as well as U.S. and international experts from industry and other stakeholder organizations participated in the meeting, which was co-hosted by CPSC and NICEATM-ICCVAM. The expert group reviewed the draft OECD TG for the LLNA: DA and proposed responses to comments from member countries. The OECD Expert Consultation convened a subsequent teleconference on December 1, 2009, to discuss outstanding issues identified at the October meeting. A revised TG was again distributed in December 2009 for review and comment to national experts and interested stakeholders of the 30 OECD member countries. A final teleconference of the OECD Expert Consultation was convened on January 29, 2010, to discuss the member country comments received during the last round of review, and a final draft TG was developed based on these discussions. This final draft was forwarded to the OECD Working Group of National Co-ordinators of the Test Guidelines Programme to consider for adoption at their March 23-25, 2010, meeting.

ICCVAM and the IWG considered the SACATM comments, the Panel report, conclusions of the OECD Expert Consultation, and all public comments before finalizing ICCVAM test method recommendations for the LLNA: DA. The recommendations (**Section 2.0**) and the final BRD (**Appendix C**) are incorporated in this ICCVAM Test Method Evaluation Report. As required by the ICCVAM Authorization Act of 2000 (Public Law 106-545, 42 United States Code 285l-3), ICCVAM will forward its recommendations to U.S. Federal agencies for consideration. Federal agencies must respond to ICCVAM within 180 days after receiving ICCVAM test method recommendations. ICCVAM recommendations are available to the public on the NICEATM-ICCVAM website, and agency responses will also be made available on the website as they are received.

[8] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E9-4280.pdf
[9] Available at http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2009.pdf
[10] Announced in 74 FR 26242 http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E9-12360.pdf

2.0 ICCVAM Recommendations for the Nonradioactive LLNA: DA Test Method

ICCVAM evaluated the validation status of the LLNA: DA as a nonradioactive modification of the traditional LLNA (ICCVAM 1999; Dean et al. 2001; Haneke et al. 2001; Sailstad et al. 2001) to identify substances that may cause ACD for regulatory hazard classification and labeling purposes. While the traditional LLNA assesses cell proliferation by measuring the incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine into the DNA of dividing cells in the draining auricular lymph nodes, the LLNA: DA assesses cell proliferation by measuring increases in ATP content in the draining auricular lymph nodes as an indicator of the cell number at the end of cell proliferation. The LLNA: DA also differs from the traditional LLNA in the test substance treatment and sampling schedule, as well as pretreatment at the application site with an aqueous solution of 1% sodium lauryl sulfate (SLS) (see **Appendix B**). NICEATM and ICCVAM prepared a comprehensive report on the data and information supporting the validity of this test method, including its accuracy and reliability compared to the traditional LLNA (see **Section 3.0** and **Appendix C)**.

2.1 ICCVAM Recommendations: Test Method Usefulness and Limitations

ICCVAM concludes that the accuracy and reliability of the LLNA: DA support use of the test method to identify substances as potential skin sensitizers and nonsensitizers. For the validation database of 44 substances,[11] the LLNA: DA correctly identified all 32 LLNA sensitizers (0% [0/32] false negatives), and nine of the 12 LLNA nonsensitizers (25% [3/12] false positives). ICCVAM recommends that a stimulation index (SI) $\geq$ 1.8 be used as the decision criterion to identify substances as potential sensitizers. ICCVAM bases this recommendation on the fact that no false negatives, relative to the traditional LLNA, result with the current validation database when an SI $\geq$ 1.8 is used.

A limitation of the LLNA: DA is the potential for false positive results when borderline positive responses between an SI of 1.8 and 2.5 are obtained (see **Section 3.4**). ICCVAM considers the applicability domain for the LLNA: DA to be the same as the traditional LLNA unless there are properties associated with a class of materials that may interfere with the accuracy of the LLNA: DA. For instance, the use of the LLNA: DA might not be appropriate for testing substances that affect ATP levels (e.g., substances that function as ATP inhibitors) or those that affect the accurate measurement of intracellular ATP (e.g., presence of ATP degrading enzymes, presence of extracellular ATP in the lymph node). In contrast, the LLNA: DA can be used for testing metal compounds, with the exception of nickel. Inconsistent results for nickel sulfate in the interlaboratory validation study suggest that the LLNA: DA may not be suitable for testing substances containing nickel and therefore further testing using a different test system is recommended when negative results are obtained for such substances.

2.2 ICCVAM Recommendations: Test Method Protocol

ICCVAM recommends a LLNA: DA test method protocol (**Appendix B**) that is based on the test method protocol developed by Yamashita et al. (2005) and Idehara et al. (2008). The ICCVAM-recommended LLNA: DA test method protocol incorporates all aspects of the ICCVAM-recommended LLNA test method protocol (Appendix A of ICCVAM 2009a) except for those procedures unique to the conduct of the LLNA: DA (**Appendix B**). Key aspects from the ICCVAM-recommended LLNA test method protocol (Appendix A of ICCVAM 2009a) included in the ICCVAM-recommended LLNA: DA test method protocol (**Appendix B**) are the following:

[11] For the accuracy analyses, results for substances tested multiple times were combined so that each substance was represented by one result. In this case, the single result used for each substance represented the most prevalent outcome. Multiple tests were available for 14 substances tested with the LLNA: DA.

- The high dose should be the maximum possible concentration (for liquids, solids, or suspensions) that does not produce systemic toxicity and/or excessive local skin irritation. The measurement of ear thickness is a potentially valuable adjunct for identifying local skin irritation.
- A minimum of four animals per dose group is recommended.
- Collection of individual animal data is recommended.
- Inclusion of a concurrent vehicle control and concurrent positive control in each study is recommended.

Additionally, ICCVAM recommends that there should be a measure of variability of the positive control response over time. Laboratories should maintain a historical database of positive control SI values such that results can be compared to the mean historical SI. There could be cause for concern when a negative test substance result is accompanied by a concurrent positive control SI value significantly lower than the mean historical SI.

In testing situations where dose-response information is not required, or negative results are anticipated, ICCVAM recommends that the reduced LLNA: DA should be considered and used where determined appropriate. The reduced LLNA: DA test method protocol uses only the high dose (Kimber et al. 2006; ESAC 2007; ICCVAM 2009b), thus further reducing animal use by up to 40%.

2.3 ICCVAM Recommendations: Future Studies

ICCVAM recommends the following future studies to further characterize the usefulness and limitations of the LLNA: DA test method:

- Efforts should be made to identify additional human data and human experience for test substances. These data may be used to further assess the usefulness and limitations of this and other versions of the LLNA for identifying human-sensitizing substances. Such efforts might include postmarketing surveillance of consumers for allergic reactions and occupational surveillance of potentially exposed workers.
- Additional substances that are nonsensitizing skin irritants should be tested to determine the impact of such substances on the false positive rate of the LLNA: DA.
- Inconsistent results for nickel sulfate suggest that the LLNA: DA may not be suitable for testing nickel compounds. Therefore, the accrual of additional data from LLNA: DA studies on such compounds with comparative human and/or guinea pig data is needed in order to more comprehensively evaluate the suitability of the LLNA: DA for testing nickel compounds.
- Efforts should be made to further characterize the sensitization potential of borderline positive substances (i.e., those that produce SI values between 1.8 and 2.5) in the LLNA: DA to determine if such results might be false positives. This could include evaluations of peptide reactivity, determination of molecular weight, identification of results from related chemicals, human studies where ethically and scientifically justified, review of occupational exposures and postmarketing experience or monitoring, or *in vitro* testing data. All decision criteria should be reassessed as additional discriminators and data become available.

2.4 ICCVAM Recommendations: Performance Standards

ICCVAM concludes that the ICCVAM-recommended performance standards (ICCVAM 2009a) for the traditional LLNA can be used to evaluate any future modifications of the LLNA: DA. The ICCVAM-recommended performance standards for the traditional LLNA apply to the LLNA: DA because the test method is functionally and mechanistically similar to the traditional LLNA. ICCVAM, in conjunction with ECVAM and JaCVAM, developed the internationally harmonized test

method performance standards for the traditional LLNA (ICCVAM 2009a) to evaluate the performance of LLNA test methods that incorporate specific protocol modifications (e.g., procedures to measure lymphocyte proliferation) compared to the traditional LLNA. Thus, unique performance standards for the LLNA: DA are not proposed at this time.

3.0 Validation Status of the LLNA: DA Test Method

The ICCVAM BRD for the LLNA: DA test method (**Appendix C**) provides a comprehensive review of the current validation status of the LLNA: DA test method, including its accuracy and reliability, the substances tested, the rationale for the standardized test method protocol used for the validation studies, and all available data supporting its validity. This section provides a brief description and summary of the validation status of the LLNA: DA test method.

3.1 Test Method Description

Originally developed by Yamashita et al. (2005) and Idehara et al. (2008), the purpose of the LLNA: DA test method is to identify potential skin sensitizers by quantifying lymphocyte proliferation. Like the traditional LLNA, the magnitude of lymphocyte proliferation measured in the LLNA: DA correlates with the extent to which sensitization develops after a topical induction exposure to a potential skin sensitizing substance.

3.1.1 General Test Method Procedures

The test substance is administered topically on days one, two, three, and seven to the dorsum of the ears of mice at a concentration that provides maximum solubility of the test substance without producing systemic toxicity and/or excessive local skin irritation. One hour prior to each test substance application, an aqueous solution of 1% SLS is applied to the dorsum of the mouse ears to increase absorption of the test substance across the skin (van Och et al. 2000). Approximately 24 hours after the last test substance administration, the draining auricular lymph nodes are excised, and a single-cell suspension from the lymph nodes of each animal is prepared for quantifying the increase in ATP content, which serves as an indicator of cell number at the end of cell proliferation.

The increase in ATP content for each mouse is measured by luciferin-luciferase assay and is expressed in relative luminescence units (RLU). The SI is calculated as the ratio of the mean RLU/mouse for each treatment group against the mean RLU/mouse for the vehicle control group. Substances producing an SI greater than a specified threshold are considered to be potential skin sensitizers. Based on the accuracy evaluation described in **Section 3.4**, the optimum accuracy was at SI $\geq$ 1.8.

3.1.2 Similarities and Differences Between the Test Method Protocols for the Traditional LLNA and the LLNA: DA

While the traditional LLNA assesses cell proliferation by measuring the incorporation of radioactive thymidine or iodine into the DNA of dividing cells in the draining auricular lymph nodes (ICCVAM 1999; Dean et al. 2001), the LLNA: DA assesses cell proliferation by measuring increases in ATP content in the draining auricular lymph nodes as an indicator of cell number at the end of cell proliferation. The LLNA: DA also differs from the traditional LLNA in the test substance treatment and sampling schedule, as well as pretreatment at the application site with an aqueous solution of 1% SLS (see **Appendix B**).

In the traditional LLNA, the test substance is topically applied on three consecutive days. Two days after the last treatment, a radioactive marker such as ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine (in phosphate-buffered saline; 250 µL/mouse) is administered via the tail vein. Then, five hours later, the draining auricular lymph nodes are excised and prepared for quantifying the incorporation of radioactivity. By comparison, in the LLNA: DA, the test substance is administered topically on days one, two, three, and seven, with each treatment preceded by application of an aqueous solution of 1% SLS. The draining auricular lymph nodes are excised 24 hrs after the last test substance application

and prepared for quantifying the increase in ATP content, which does not require injection of a marker chemical.

3.2 Validation Database

The current validation database for the LLNA: DA includes results from studies for 46 substances that had previously been tested in the traditional LLNA. The LLNA: DA results were obtained from either the intralaboratory (Idehara et al. 2008; unpublished data) and/or the two-phased interlaboratory (Omori et al. 2008) validation study. These data were available and reviewed by the Panel in April 2009.

The reference test data for the 46 substances were obtained from traditional LLNA tests. Of the 46 substances, 33 were classified by the traditional LLNA as skin sensitizers, 12 were classified as nonsensitizers, and one (benzocaine) was classified as equivocal due to highly variable results (Basketter et al. 1995; ICCVAM 1999) and was not included in the performance analyses. Similar to benzocaine, traditional LLNA data for toluene 2,4-diisocyanate (van Och et al. 2000) were not suitable for comparison (i.e., a modified version of the traditional LLNA test method protocol was used that was not in accordance with OECD TG 429 [OECD 2002] or ICCVAM 1999 and Dean et al. 2001) and results for this test substance were not included in the performance analysis. Thus, the validation database is comprised of 44 substances tested in the LLNA: DA that have adequate traditional LLNA reference data for use in the performance analyses. Results from guinea pig skin sensitization testing and human skin sensitization testing and/or published clinical case report information are also provided where they were available (see **Appendix C, Annex III**). Of the 46 substances, 42 had guinea pig skin sensitization testing data and 43 had human skin sensitization testing data and/or published clinical case report information. Similar to LLNA: DA comparisons with the traditional LLNA, benzocaine and toluene 2,4-diisocyanate were not included in comparisons between the LLNA: DA and guinea pig or human outcomes.

Table 3-1 lists the chemical classifications, traditional LLNA EC3 values with maximum SI values, and LLNA: DA EC1.8 values with maximum SI values for the 44 substances with adequate comparative LLNA data that were evaluated in the LLNA: DA performance analyses. Twenty chemical classes were represented by the 44 substances evaluated in the LLNA: DA performance analyses; 13 substances were classified in more than one chemical class. The classes with the highest number of substances were carboxylic acids (16 substances) and phenols (5 substances). Further, of the 22 chemical classes represented in the NICEATM LLNA database by at least five substances (thereby providing a sufficiently large representation for further analyses), 20 classes had at least 60% of the traditional LLNA results identified as positive. For this database of more than 600 substances, these classes were identified as those most likely to be associated with skin sensitization. Seventeen of these classes were also represented in the LLNA: DA database (only amides, ketones, and macromolecular substances were not included). Among the chemical classes that have been previously identified as common skin allergens (e.g., aldehydes, ketones, quinones, and acrylates, [Gerberick et al. 2004]), only ketones were not included in the LLNA: DA database. Nevertheless, the Panel considered the database of substances tested in the LLNA: DA to be representative of a sufficient range of chemicals typically tested for skin sensitization potential. The traditional LLNA EC3 values (i.e., estimated concentration needed to produce an SI = 3) for the 32 sensitizers ranged from 0.009% to 90%.

Table 3-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: DA EC1.8 Values, and Maximum SI Values for 44 Substances Evaluated in the LLNA: DA Performance Analyses

Substance Name	Product Use[1]	Chemical Class[2]	Trad. LLNA EC3 (%) (Max. SI)[3]	LLNA: DA EC1.8 (%) (Max. SI)[3]
5-Chloro-2-methyl-4-isothiazolin-3-one[4]	Cosmetics; Manufacturing; Pesticides	Sulfur Compounds; Heterocyclic Compounds	0.009 (27.7)	0.009 (7.5)
p-Benzoquinone[4]	Manufacturing; Pesticides; Pharmaceuticals	Quinones	0.010 (52.3)	0.003 (3.8)
2,4-Dinitrochlorobenzene[5, 6]	Manufacturing; Pesticides	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated; Nitro Compounds	0.049 (43.9)	0.032 (15.1)
Benzalkonium chloride[5]	Cosmetics; Disinfectant; Manufacturing; Personal care products; Pesticides	Amines; Onium Compounds	0.070[7] (11.1)	0.402 (6.7)
Glutaraldehyde[5, 6]	Cosmetics; Disinfectant; Manufacturing; Pesticides	Aldehydes	0.083 (18.0)	0.118 (6.5)
p-Phenylenediamine[5]	Intermediate in chemical synthesis; Manufacturing	Amines	0.110 (26.4)	0.036 (5.1)
Potassium dichromate[5, 8]	Manufacturing; Pharmaceuticals	Inorganic Chemical, Chromium Compounds; Inorganic Chemical, Potassium Compounds	0.170 (33.6)	0.062 (6.4)
Propyl gallate[4]	Cosmetics; Food additive	Carboxylic Acids	0.320 (33.6)	0.225 (5.0)
Phthalic anhydride[5]	Intermediate in chemical synthesis; Manufacturing; Pharmaceuticals	Anhydrides; Carboxylic Acids	0.360 (26.0)	0.030 (6.9)
Formaldehyde[5, 6]	Disinfectant; Manufacturing	Aldehydes	0.495 (4.0)	0.699 (5.1)
Cobalt chloride[5, 6, 8]	Manufacturing; Pesticides	Inorganic Chemical, Elements; Inorganic Chemical, Metals	0.600 (7.2)	0.859 (20.6)
Isoeugenol[5, 6]	Food additive; Fragrance agent	Carboxylic Acids	1.540 (31.0)	1.477 (12.4)

continued

Table 3-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: DA EC1.8 Values, and Maximum SI Values for 44 Substances Evaluated in the LLNA: DA Performance Analyses (continued)

Substance Name	Product Use[1]	Chemical Class[2]	Trad. LLNA EC3 (%) (Max. SI)[3]	LLNA: DA EC1.8 (%) (Max. SI)[3]
2-Mercaptobenzothiazole[5]	Manufacturing; Pesticides	Heterocyclic Compounds	1.700 (8.6)	7.992 (2.0)
Cinnamic aldehyde[5]	Cosmetics; Food additive; Fragrance agent; Intermediate in chemical synthesis; Personal care products; Pesticides	Aldehydes	1.910 (18.4)	0.635 (4.7)
3-Aminophenol[6]	Cosmetics; Pharmaceuticals	Amines; Phenols	3.200 (5.7)	1.841 (2.8)
Diethyl maleate[4]	Food additive; Intermediate in chemical synthesis	Carboxylic Acids	3.600 (22.6)	0.442 (3.8)
Trimellitic anhydride[5]	Manufacturing	Anhydride; Carboxylic Acids	4.710 (4.6)	0.058 (5.0)
Nickel (II) sulfate hexahydrate[5, 6, 8]	Manufacturing	Inorganic Chemical, Elements; Inorganic Chemical, Metals	4.800 (3.1)	2.606 (11.8)
Resorcinol[5]	Cosmetics; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Phenols	6.330 (10.4)	3.902 (4.3)
Sodium lauryl sulfate[5]	Cosmetics; Food additive; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Alcohols; Sulfur Compounds; Lipids	8.080 (8.9)	1.640 (3.4)
Citral[5]	Fragrance agent	Hydrocarbons, Other	9.170 (20.5)	2.053 (4.4)
Hexyl cinnamic aldehyde[5, 6, 8]	Food additive; Fragrance agent	Aldehydes	9.740 (20.0)	6.275 (10.2)

continued

Table 3-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: DA EC1.8 Values, and Maximum SI Values for 44 Substances Evaluated in the LLNA: DA Performance Analyses (continued)

Substance Name	Product Use[1]	Chemical Class[2]	Trad. LLNA EC3 (%) (Max. SI)[3]	LLNA: DA EC1.8 (%) (Max. SI)[3]
Eugenol[5]	Cosmetics; Food additive; Intermediate in chemical synthesis; Manufacturing; Personal care products; Pharmaceuticals	Carboxylic Acids	10.090 (17.0)	2.629 (7.1)
Abietic acid[5, 6]	Manufacturing	Hydrocarbons, Cyclic; Polycyclic Compounds	11.920 (5.2)	4.530 (8.0)
Phenyl benzoate[4]	Manufacturing; Pesticides	Carboxylic Acids	13.600 (11.1)	0.653 (4.2)
Cinnamic alcohol[4]	Cosmetics; Food additive; Fragrance agent; Intermediate in chemical synthesis; Personal care products	Alcohols	21.000 (5.7)	5.218 (5.7)
Hydroxycitronellal[5]	Food additive; Fragrance agent; Personal care products	Hydrocarbons, Other	23.750 (8.5)	8.674 (5.7)
Imidazolidinyl urea[5]	Cosmetics; Personal care products; Pesticides	Urea	24.000 (5.5)	6.275 (4.7)
Ethylene glycol dimethacrylate[4]	Manufacturing	Carboxylic Acids	28.000 (7.0)	19.236 (4.5)
Butyl glycidyl ether[4]	Intermediate in chemical synthesis; Manufacturing	Ethers	30.900 (5.6)	17.507 (4.6)
Ethyl acrylate[4]	Manufacturing	Carboxylic Acids	32.800 (4.0)	6.790 (4.3)
Methyl methacrylate[4]	Manufacturing	Carboxylic Acids	90.000 (3.6)	99.347 (1.8)
1-Bromobutane[5]	Intermediate in chemical synthesis; Pharmaceuticals; Solvent	Hydrocarbons, Halogenated	NA (1.2)	NA (1.7)

continued

Table 3-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: DA EC1.8 Values, and Maximum SI Values for 44 Substances Evaluated in the LLNA: DA Performance Analyses (continued)

Substance Name	Product Use[1]	Chemical Class[2]	Trad. LLNA EC3 (%) (Max. SI)[3]	LLNA: DA EC1.8 (%) (Max. SI)[3]
Chlorobenzene[5]	Manufacturing; Solvent	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated	NA (1.7)	17.877 (2.4)
Diethyl phthalate[5]	Cosmetics; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Carboxylic Acids	NA (1.5)	NA (1.1)
Dimethyl isophthalate[4, 6]	Manufacturing; Fragrance agent	Carboxylic Acids	NA (1.0)	NA (1.3)
Hexane[5]	Manufacturing; Solvent	Hydrocarbons, Acyclic	NA (2.2)	82.232 (2.3)
Isopropanol[5, 6]	Cosmetics; Disinfectant; Food additive; Intermediate in chemical synthesis; Manufacturing; Personal care products; Pharmaceuticals; Solvent	Alcohols	NA (1.7)	NA (2.0)
Lactic acid[5, 8]	Food additive; Manufacturing; Pharmaceuticals	Carboxylic Acids	NA (2.2)	NA (1.1)
Methyl salicylate[5, 6]	Cosmetics; Food additive; Fragrance agent; Personal care products; Pharmaceuticals; Solvent	Carboxylic Acids; Phenols	NA (2.9)	NA (1.8)
Propylparaben[5]	Food additive; Pesticides; Pharmaceuticals	Carboxylic Acids; Phenols	NA (1.4)	NA (1.3)
Nickel (II) chloride[4]	Manufacturing; Pesticides	Inorganic Chemical, Elements; Inorganic Chemical, Metals	NA (2.4)	NA (1.3)
Salicylic acid[4]	Food additive; Manufacturing; Pharmaceuticals	Phenols; Carboxylic Acids	NA (2.5)	17.768 (2.0)

continued

Table 3-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: DA EC1.8 Values, and Maximum SI Values for 44 Substances Evaluated in the LLNA: DA Performance Analyses (continued)

Substance Name	Product Use[1]	Chemical Class[2]	Trad. LLNA EC3 (%) (Max. SI)[3]	LLNA: DA EC1.8 (%) (Max. SI)[3]
Sulfanilamide[4]	Pharmaceuticals	Hydrocarbons, Cyclic; Sulfur Compounds	NA (1.0)	NA (0.9)

Abbreviations: EC3 = estimated concentration needed to produce a stimulation index of three; EC1.8 = estimated concentration needed to produce a stimulation index of 1.8; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; Max. = maximum; NA = not available; SI = stimulation index.

[1] Information for product use was gathered from the following databases:
Hazardous Substances Database - National Library of Medicine – TOXNET: http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB
Haz-Map: National Library of Medicine-Toxicology and Environmental Health Information Program: http://hazmap.nlm.nih.gov/
Household Products Database - National Library of Medicine: http://hpd.nlm.nih.gov/index.htm
International Programme on Chemical Safety INCHEM database in partnership with Canadian Centre for Occupational Health and Safety: http://www.inchem.org/
National Toxicology Program: http://ntp.niehs.nih.gov:8080/index.html?col=010stat

[2] Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, as developed by the National Library of Medicine: http://www.nlm.nih.gov/mesh/meshhome.html.

[3] The traditional LLNA EC3 or LLNA: DA EC1.8 values listed for each substance is averaged from respective studies. The substance was tested in the same vehicle in both the traditional LLNA and the LLNA: DA, except where noted. Numbers in parentheses indicate the maximum SI.

[4] Substance tested in the intralaboratory validation study (Idehara unpublished).

[5] Substance tested in the intralaboratory validation study (Idehara et al. 2008).

[6] Substance tested in phase one of the two-phased interlaboratory validation study (Omori et al. 2008).

[7] Benzalkonium chloride was tested in the LLNA: DA using acetone: olive oil (4:1) as the vehicle but the traditional LLNA EC3 value reported is based on results using acetone as the vehicle.

[8] Substance tested in phase two of a two-phased interlaboratory validation study (Omori et al. 2008).

Annex II of the BRD (**Appendix C**) lists various physicochemical properties for the substances tested in the LLNA: DA. For the 44 substances that were evaluated in the LLNA: DA performance analyses, the molecular weights ranged from 30 to 388 g/mol. Twenty-two of the 44 substances were solids, 21 were liquids, and one substance (benzalkonium chloride) exists as either a solid or a liquid. The estimated log octanol-water partition coefficients (K_{ow}) were available for 38 substances and ranged from -8.28 to 6.46. Peptide reactivity, which was available for 28 substances, ranged from high to minimal (Gerberick et al. 2004, 2007).

3.3 Reference Test Method Data

The traditional LLNA reference data used for the accuracy analyses were from ICCVAM (1999) for 34 of the 44 substances that were evaluated. The traditional LLNA reference data for the remaining 10 substances were obtained from the scientific literature (Gerberick et al. 1992; Hilton et al. 1998; Ryan et al. 2002; Basketter et al. 2005; Gerberick et al. 2005; Betts et al. 2006; Basketter et al. 2007). The reference data for the guinea pig tests (GPMT or Buehler test) and human tests (human maximization test, human patch test allergen, or other human data) were also obtained from the scientific literature. The LLNA, guinea pig, and human reference data and their sources for each of the 44 substances evaluated are provided in Annex III of the BRD (**Appendix C**).

3.4 Test Method Accuracy

The ICCVAM evaluation of the LLNA: DA included an assessment of multiple decision criteria (see **Table 3-2**) including SI ≥ 3.0, the threshold for distinguishing sensitizers and nonsensitizers that is recommended in the LLNA: DA developer's test method protocol. When the optimal decision criterion of SI ≥ 1.8 was used to identify sensitizers vs. nonsensitizers, compared to the traditional LLNA, accuracy was 93% (41/44), with a false positive rate of 25% (3/12), and a false negative rate of 0% (0/32). All three false positive substances were tested once in the LLNA: DA and had resulting maximum SI values between 1.8 and 2.5 (chlorobenzene maximum SI = 2.44; hexane maximum SI = 2.31; salicylic acid maximum SI = 2.00). Other available information, such as dose-response, evidence of systemic toxicity or excessive local irritation, and (where appropriate) statistical significance together with SI values should be considered to confirm that such borderline positive results are potential skin sensitizers. Consideration should also be given to various properties of the test substance, including whether it is structurally similar to known skin sensitizers. For example, peptide reactivity (Gerberick et al. 2007), could be used to interpret LLNA: DA results when borderline positive results (e.g., SI values between 1.8 and 2.5) are produced to confirm that such results are not false positive. Two of the three traditional LLNA nonsensitizers with positive LLNA: DA SI values in this range had minimal peptide reactivity and one did not have peptide reactivity data available. No unique characteristics were identified that could be used as rationale for excluding any particular types of substances from testing in the LLNA: DA.

An evaluation to determine the robustness of the optimum SI ≥ 1.8 criterion indicated that the SI was quite stable. Taking different samples of the data as training and validation sets had relatively little impact on the cutoff SI criterion or on the resulting number of false or false negative results.

Table 3-2 Performance of the LLNA: DA for 44 Substances Compared to the Traditional LLNA in Predicting Skin Sensitization Potential Using Alternative Decision Criteria Based on the Most Prevalent Outcome for Substances with Multiple Tests

Alternate Criterion	N[1]	Accuracy % (No.[2])	Sensitivity % (No.[2])	Specificity % (No.[2])	False Positive Rate % (No.[2])	False Negative Rate % (No.[2])	Positive Predictivity % (No.[2])	Negative Predictivity % (No.[2])
Statistics[3]	44	84 (37/44)	94 (30/32)	58 (7/12)	42 (5/12)	6 (2/32)	86 (30/35)	78 (7/9)
≥95% CI[4]	44	75 (33/44)	100 (32/32)	8 (1/12)	92 (11/12)	0 (0/32)	74 (32/43)	100 (1/1)
≥2 SD[5]	44	77 (34/44)	91 (29/32)	42 (5/12)	58 (7/12)	9 (3/32)	81 (29/36)	63 (5/8)
≥3 SD[6]	44	80 (35/44)	88 (28/32)	58 (7/12)	42 (5/12)	13 (4/32)	85 (28/33)	64 (7/11)
SI ≥ 5.0	44	57 (25/44)	41 (13/32)	100 (12/12)	0 (0/12)	59 (19/32)	100 (13/13)	39 (12/31)
SI ≥ 4.5	44	70 (31/44)	59 (19/32)	100 (12/12)	0 (0/12)	41 (13/32)	100 (19/19)	48 (12/25)
SI ≥ 4.0	44	84 (37/44)	78 (25/32)	100 (12/12)	0 (0/12)	22 (7/32)	100 (25/25)	63 (12/19)
SI ≥ 3.5	44	89 (39/44)	84 (27/32)	100 (12/12)	0 (0/12)	16 (5/32)	100 (27/27)	71 (12/17)
SI ≥ 3.0	*44*	*91 (40/44)*	*88 (28/32)*	*100 (12/12)*	*0 (0/12)*	*13 (4/32)*	*100 (28/28)*	*75 (12/16)*
SI ≥ 2.5	44	91 (40/44)	88 (28/32)	100 (12/12)	0 (0/12)	13 (4/32)	100 (28/28)	75 (12/16)
SI ≥ 2.0	44	91 (40/44)	97 (31/32)	75 (9/12)	25 (3/12)	3 (1/32)	91 (31/34)	90 (9/10)
SI ≥ 1.8	**44**	**93 (41/44)**	**100 (32/32)**	**75 (9/12)**	**25 (3/12)**	**0 (0/32)**	**91 (32/35)**	**100 (9/9)**
SI ≥ 1.5	44	89 (39/44)	100 (32/32)	58 (7/12)	42 (5/12)	0 (0/32)	86 (32/37)	100 (7/7)
SI ≥ 1.3	44	86 (38/44)	100 (32/32)	50 (6/12)	50 (6/12)	0 (0/32)	84 (32/38)	100 (6/6)

Italicized text indicates the decision criterion chosen by the LLNA: DA validation study team; Bolded text indicates the single decision criterion that had an overall increased performance in predicting skin sensitization potential when compared to the traditional LLNA.

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; No. = number; SD = standard deviation; SI = stimulation index.

[1] N = Number of substances included in this analysis.

[2] The proportion on which the percentage calculation is based.

[3] Analysis of variance for difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The ATP data were log-transformed prior to statistical analysis. For analysis of variance, significance at $p < 0.05$ was further tested by Dunnett's test.

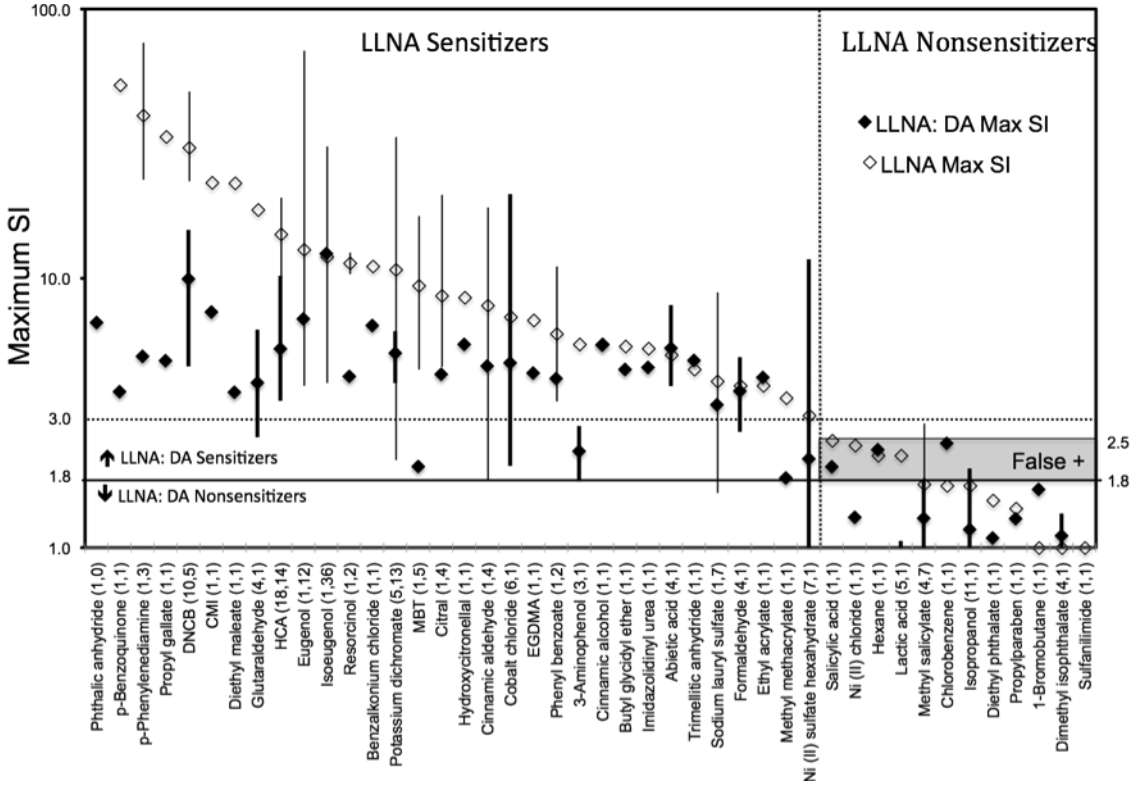
[4] The mean ATP of at least one treatment group was outside the 95% confidence interval for the mean ATP of the vehicle control group.

[5] The mean ATP of at least one treatment group was greater than 2 SD from the mean ATP of the vehicle control group.

[6] The mean ATP of at least one treatment group was greater than 3 SD from the mean ATP of the vehicle control group.

Figure 3-1 shows that SI values for the LLNA: DA are generally lower than those for traditional LLNA tests at similar test doses. SI values for substances with more than one test result are represented by the geometric mean with bars to show the overall range of individual study results used to calculate the geometric mean. The purpose of showing the geometric mean and associated ranges is to provide an assessment of variability among results, and the relative sensitivity of the traditional LLNA and LLNA: DA results. However, the accuracy analyses reported in the BRD are based on individual test results and not on a geometric mean. **Table 3-3** lists the maximum SI values for the substances included in **Figure 3-1**.

Figure 3-1 Comparison of LLNA: DA Stimulation Index with Traditional LLNA Stimulation Index[1]



Abbreviations: CMI = 5-chloro-2-methyl-4-isothiazolin-3-one; DNCB = 2,4-dinitrochlorobenzene; EGDMA = ethylene glycol dimethacrylate; HCA = hexyl cinnamic aldehyde; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; MBT = 2-mercaptobenzothiazole; Ni = nickel; False + = false positive results in the LLNA: DA based on majority call were in the SI range between 1.8 and 2.5; SI = stimulation index.

[1] LLNA: DA and traditional LLNA tests at similar doses are shown. Symbols show the maximum SI for substances with one test result or geometric mean maximum SI for substances with more than one test result. Bars show the range of values reported for multiple test results (heavy bars for LLNA: DA and light bars for traditional LLNA). Numbers in parentheses beside the substance names indicate the number of tests for the LLNA: DA followed by the traditional LLNA, which may differ from the total number of tests available since only tests with similar maximum doses were used in this figure. The accuracy analyses used individual test results rather than geometric mean SI values. Using individual test results, traditional LLNA nonsensitizers with at least one positive LLNA: DA test result in the SI range between 1.8 and 2.5 include salicylic acid, hexane, chlorobenzene, and isopropanol.

Table 3-3 Maximum SI Values of 44 Substances Evaluated in the LLNA: DA Compared to Traditional LLNA Tests with Similar Doses[1]

Substance Name[2]	Test Vehicle[3]	LLNA: DA Maximum SI Values[4]	Traditional LLNA Maximum SI Values
Sensitizers (LLNA: DA SI ≥ 1.8 and Traditional LLNA SI ≥ 3.0)			
Phthalic anhydride (1, 0)	AOO	6.85	NA
p-Benzoquinone (1, 1)	AOO	3.79	52.30
p-Phenylenediamine (1, 3)	AOO	5.14	23.30, 37.40, 75.30
Propyl gallate (1, 1)	AOO	4.95	33.60
DNCB (10, 5)	AOO	4.71, 7.86, 8.53, 9.23, 9.96, 10.89, 11.97, 12.60, 13.18, 15.14	23.00, 24.00, 26.80, 36.70, 49.60
CMI (1, 1)	DMF	7.50	22.70
Diethyl maleate (1, 1)	AOO	3.78	22.60
Glutaraldehyde (4, 1)	ACE	2.57, 3.39, 5.00, 6.45	18.00
HCA (18, 14)	AOO	3.51, 3.88, 3.92, 3.97, 4.44, 4.47, 4.82, 5.11, 5.41, 5.50, 5.71, 5.78, 6.45, 6.47, 7.09, 7.60, 8.42, 10.22	10.00, 11.60, 11.60, 13.40, 14.00, 14.00, 14.10, 14.50, 16.00, 17.00, 17.00, 17.00, 17.60, 20.00
Eugenol (1, 12)	AOO	7.07	4.01, 6.10, 9.30, 9.60, 10.20, 12.40, 14.10, 16.00, 16.10, 16.10, 17.00, 70.30
Isoeugenol (1, 36)	AOO	12.36	4.10, 4.90, 5.00, 5.60, 6.70, 6.80, 7.20, 7.20, 7.50, 7.50, 7.60, 8.70, 10.00, 11.00, 11.10, 11.80, 12.40, 13.80, 13.10, 13.10, 13.10, 14.10, 14.70, 14.70, 15.30, 17.00, 18.40, 19.00, 23.20, 19.20, 19.30, 23.20, 23.60, 24.40, 29.80, 31.00
Resorcinol (1, 2)	AOO	4.33	10.40, 12.50
Benzalkonium chloride (1, 1)	AOO / ACE	6.68	11.10
Potassium dichromate (5, 13)	DMSO	4.08, 4.78, 5.49, 6.01, 6.37	2.12, 5.40, 6.90, 10.10, 10.10, 10.40, 11.20, 13.00, 13.10, 16.10, 16.10, 19.10, 33.60
Citral (1, 4)	AOO	4.40	4.70, 6.20, 9.30, 20.50
Hydroxycitronellal (1, 1)	AOO	5.69	8.50
Cinnamic aldehyde (1, 4)	AOO	4.73	1.80, 7.60, 15.80, 18.40
EGDMA (1, 1)	MEK	4.45	7.00
Phenyl benzoate (1, 2)	AOO	4.24	3.50, 11.10

continued

Table 3-3 Maximum SI Values of 44 Substances Evaluated in the LLNA: DA Compared to Traditional LLNA Tests with Similar Doses[1] (continued)

Substance Name[2]	Test Vehicle[3]	LLNA: DA Maximum SI Values[4]	Traditional LLNA Maximum SI Values
Sensitizers (LLNA: DA SI ≥ 1.8 and Traditional LLNA SI ≥ 3.0)			
Cinnamic alcohol (1, 1)	AOO	5.66	5.70
Butyl glycidyl ether (1, 1)	AOO	4.59	5.60
Imidazolidinyl urea (1, 1)	DMF	4.67	5.50
Abietic acid (4, 1)	AOO	3.98, 4.64, 6.26, 7.96	5.20
Trimellitic anhydride (1, 1)	AOO	4.96	4.60
Sodium lauryl sulfate (1, 7)	DMF	3.39	1.60, 2.60, 4.10, 5.10, 5.10, 5.40, 8.90
Formaldehyde (4, 1)	ACE	2.69, 3.18, 4.84, 5.10	4.00
Ethyl acrylate (1, 1)	AOO	4.29	3.98
MBT (1, 5)	DMF	**2.00**	4.60, 9.10, 9.50, 10.80, 17.10
Cobalt chloride (6, 1)	DMSO	**2.01**, 2.54, 3.64, 4.25, 8.07, 20.55	7.21
3-Aminophenol (3, 1)	AOO	1.76, **2.38, 2.83**	5.70
Methyl methacrylate (1, 1)	AOO	**1.81**	3.60
Ni (II) sulfate hexahydrate (7, 1)	DMSO	0.79, 1.24, 1.52, 1.56, **2.13**, 3.49, 11.78	3.10
Traditional LLNA Nonsensitizers (SI < 3.0) with Borderline Positive SI Values in LLNA: DA (1.8 < SI <2.5; see bold text)			
Salicylic acid (1, 1)	AOO	**2.00**	2.50
Hexane (1, 1)	AOO	**2.31**	2.20
Chlorobenzene (1, 1)	AOO	**2.44**	1.70
Nonsensitizers (LLNA: DA SI < 1.8 and Traditional LLNA SI < 3.0)			
Ni (II) chloride (1, 1)	DMSO	1.30	2.40
Lactic acid (5, 1)	DMSO	0.91, 0.93, 0.97, 0.99, 1.06	2.20
Methyl salicylate (4, 7)	AOO	0.83, 1.20, 1.55, 1.77	0.90, 1.10, 1.72, 1.90, 2.10, 2.30, 2.90
Isopropanol (11, 1)	AOO	0.70, 0.76, 0.91, 1.01, 1.08, 1.21, 1.25, 1.45, 1.54, 1.57, **1.97**	1.70
Diethylphthalate (1, 1)	AOO	1.09	1.50
Propylparaben (1, 1)	AOO	1.28	1.40
1-Bromobutane (1, 1)	AOO	1.65	1.00

continued

Table 3-3 Maximum SI Values of 44 Substances Evaluated in the LLNA: DA Compared to Traditional LLNA Tests with Similar Doses[1] (continued)

Substance Name[2]	Test Vehicle[3]	LLNA: DA Maximum SI Values[4]	Traditional LLNA Maximum SI Values
Nonsensitizers (LLNA: DA SI < 1.8 and Traditional LLNA SI < 3.0)			
Dimethyl isophthalate (4, 1)	AOO	0.89, 1.00, 1.26, 1.34	1.00
Sulfanilimide (1, 1)	DMF	0.86	1.00

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); CMI = 5-Chloro-2-methyl-4-isothiazolin-3-one; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; DNCB = 2,4-dinitrochlorobenzene; EGDMA = ethylene glycol dimethacrylate; HCA = hexyl cinnamic aldehyde; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; MBT = 2-mercaptobenzothiazole; MEK = methyl ethyl ketone; NA = not available; Ni = nickel; SI = stimulation index.

[1] LLNA: DA and traditional LLNA tests at similar doses are shown and correspond to the same data depicted in **Figure 3-1**.

[2] Numbers in parentheses beside the substance names indicate the number of tests for the LLNA: DA followed by the traditional LLNA, which may differ from the total number of tests available since only tests with similar doses were included.

[3] The vehicle used was the same in LLNA: DA and traditional LLNA tests except for one substance, and in this case (for benzalkonium chloride) the first entry is the vehicle used for the LLNA: DA, and the second entry is for the traditional LLNA.

[4] The bold text indicates LLNA: DA tests with maximum SI values between 1.8 and 2.5.

3.5 Test Method Reliability (Intra- and Interlaboratory Reproducibility)

The BRD details the evaluation of intra- and interlaboratory reproducibility of the LLNA: DA test method (see **Section 7.0** of **Appendix C**). Intralaboratory reproducibility was assessed using a coefficient of variation (CV) analysis of EC3 (estimated concentration needed to produce an SI of 3.0) and EC1.8 values (estimated concentration needed to produce an SI of 1.8) for isoeugenol and eugenol (each substance was tested in three different experiments). The mean EC3 values and corresponding CVs for isoeugenol and eugenol were 2.74% ± 0.58% with a 21% CV, and 5.06% ± 0.55%, with an 11% CV, respectively. The mean EC1.8 values and corresponding CVs for isoeugenol and eugenol were 0.87% ± 0.31% (36% CV), and 3.38% ± 0.79% (23% CV), respectively.

Qualitative analyses of LLNA: DA reproducibility were conducted in both phases of an interlaboratory validation study, using SI ≥ 1.8 as the threshold to distinguish sensitizers from nonsensitizers. In the first phase (n = 12 substances [nine sensitizers and three nonsensitizers based on traditional LLNA test results] tested in three or 10 laboratories) there was 100% agreement among the laboratories for 10 substances (seven sensitizers and three nonsensitizers based on traditional LLNA test results). There was 67% (2/3) agreement among the tests for the remaining two traditional LLNA sensitizers. The interlaboratory CV values for the EC1.8 values for eight of the nine traditional LLNA sensitizers ranged from 15% to 140%. The interlaboratory CV value for the EC1.8 values for the traditional LLNA sensitizer nickel (II) sulfate hexahydrate could not be calculated since an EC1.8 value was only available from one of the three laboratories that tested it.

In the second phase (n = 5 substances [four sensitizers and one nonsensitizer based on traditional LLNA test results] tested in four or seven laboratories) there was 100% agreement among the

laboratories for four substances (three sensitizers and one nonsensitizer based on traditional LLNA results). There was 75% (3/4) agreement among the tests for the remaining traditional LLNA sensitizer. Interlaboratory CV values for the EC1.8 values of the four traditional LLNA sensitizers ranged from 14% to 93%.

There were 14 substances with multiple tests across the two phases of the interlaboratory validation study that could be used for analyses of reproducibility when using SI ≥ 1.8 to identify potential sensitizers. The SI results for 80% (8/10) of the sensitizers (based on traditional LLNA results) were 100% concordant in the LLNA: DA (i.e., all tests for that substance yielded maximum SI ≥ 1.8) (**Table 3-4**). The two traditional LLNA sensitizers with LLNA: DA tests that yielded maximum SI values less than 1.8 were 3-aminophenol and nickel (II) sulfate hexahydrate. The SI results for 75% (3/4) of the nonsensitizers (based on traditional LLNA results) were 100% concordant in the LLNA: DA (i.e., all tests for that substance yielded SI < 1.8). The concordance of the other nonsensitizer, isopropanol, was 91% (10/11).

Table 3-4 Concordance of LLNA: DA Tests for Substances with Multiple Tests Based on Maximum SI Category

Substance Name	LLNA: DA Nonsensitizers (Maximum SI < 1.8)[1]	LLNA: DA Sensitizers (SI ≥ 1.8)		Total Tests
		1.8 < Maximum SI < 2.5[1]	Maximum SI ≥ 2.5[1]	
Sensitizers[2]				
Abietic acid	0 (0%)	0 (0%)	4 (100%)	4
3-Aminophenol	1 (33.3%)	1 (33.3%)	1 (33.3%)	3
Cobalt chloride	0 (0%)	1 (12.5%)	7 (87.5%)	8
2,4-Dinitrochlorobenzene	0 (0%)	0 (0%)	11 (100%)	11
Formaldehyde	0 (0%)	0 (0%)	4 (100%)	4
Glutaraldehyde	0 (0%)	0 (0%)	4 (100%)	4
Hexyl cinnamic aldehyde	0 (0%)	0 (0%)	18 (100%)	18
Isoeugenol	0 (0%)	0 (0%)	4 (100%)	4
Nickel (II) sulfate hexahydrate	4 (50%)	2 (25%)	2 (25%)	8
Potassium dichromate	0 (0%)	0 (0%)	5 (100%)	5
Nonsensitizers[2]				
Dimethyl isophthalate	4 (100%)	0 (0%)	0 (0%)	4
Isopropanol	10 (91%)	1 (9%)	0 (0%)	11
Lactic acid	5 (100%)	0 (0%)	0 (0%)	5
Methyl salicylate	4 (100%)	0 (0%)	0 (0%)	4

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] Numbers shown reflect number of tests. Percentage in parentheses reflects percentage of the total number of tests for each substance.

[2] Based on traditional LLNA test results.

3.6 Animal Welfare Considerations: Reduction, Refinement, and Replacement

The LLNA: DA will use the same number of animals as the updated ICCVAM-recommended traditional LLNA test method protocol (Appendix A of ICCVAM 2009a). However, since use of the traditional LLNA is restricted in some countries and institutions because of limitations on handling radioactivity, availability and use of the nonradioactive LLNA: DA may lead to further reduction in use of the guinea pig tests, which would provide for reduced animal use and increased refinement by avoiding the discomfort that can occur in the guinea pig tests when substances cause ACD. Additionally, the LLNA: DA test method protocol requires fewer mice per treatment group (a minimum of four animals per group) than either of the guinea pig tests (10-20 animals/group for the Buehler test and 5-10 animals/group for the GPMT).

4.0 ICCVAM Consideration of Independent Peer Review Panel Report and Other Comments

The ICCVAM evaluation process incorporates a high level of scientific peer review and transparency. The evaluation process for the LLNA: DA included two public review meetings by an independent scientific peer review panel, multiple opportunities for public comments (see **Section 1.0**), consideration of reports from an OECD Expert Consultation, and comments from the SACATM. ICCVAM and the IWG considered the Panel report, conclusions of the OECD Expert Consultation, the SACATM comments, and all public comments before finalizing the ICCVAM Test Method Evaluation Report and final BRD for the LLNA: DA. This section summarizes the ICCVAM consideration of these reports and comments. The Panel reports and public comments are provided in **Appendices D** and **F**.

4.1 ICCVAM Consideration of Independent Peer Review Panel Report and OECD Comments

4.1.1 Comments on Revised Draft ICCVAM Recommendations: Test Method Usefulness and Limitations

The Panel agreed that the available data and test method performance supported the use of the LLNA: DA to identify substances as potential sensitizers and nonsensitizers, with certain limitations. The Panel noted that the accuracy analysis they reviewed supported using two decision criteria (i.e., one to identify sensitizers and one to identify nonsensitizers). The Panel emphasized that the decision criteria were empirically derived from the data and produced the best combination of maximum accuracy coupled with the minimum number of results in the range of uncertainty (i.e., the range in which maximum SI results were between the decision criteria for sensitizers and nonsensitizers). Since using two decision criteria allows for a more definitive identification of sensitizers and nonsensitizers, this approach provides animal welfare benefits by reducing further tests that might be required in instances where the hazard classification of a substance is not as clear. In addition, one can use statistical analysis and/or other data and information (e.g., peptide reactivity, quantitative structure-activity relationships, skin penetration information) to provide more information on compounds that fall in the range of uncertainty. However, the Panel questioned how results in the range of uncertainty would be useful for regulatory purposes and emphasized that additional guidance would be needed on how to classify substances with SI values in the range of uncertainty.

The OECD Expert Consultation viewed that despite certain limitations, the LLNA: DA is useful as a modified LLNA test method that has the potential to reduce the number of animals required and refine the way in which animals are used for ACD testing. Like the Panel, OECD member country experts questioned the regulatory utility of the LLNA: DA since specific guidance on how to classify substances with SI values in the range of uncertainty has yet to be developed. Therefore, they recommended instead that a single decision criterion (as was originally proposed by ICCVAM and reviewed by the Panel in 2008) would be more useful to identify substances as potential sensitizers. They agreed with ICCVAM that SI $\geq$ 1.8 provided optimal test method performance by preventing false negative results. They also agreed with ICCVAM that users may want to consider additional information such as dose-response, evidence of systemic toxicity and/or excessive local skin irritation, and (where appropriate) statistical significance together with SI values to confirm borderline positive results (i.e., SI between 1.8 and 2.5) as potential skin sensitizers. Additionally, the OECD Expert Consultation agreed that the use of the LLNA: DA might not be appropriate for testing substances that affect ATP levels (e.g., substances that function as ATP inhibitors) or those that affect the accurate measurement of intracellular ATP (e.g., presence of ATP degrading enzymes, presence of extracellular ATP in the lymph node).

ICCVAM considered the Panel report and the OECD Expert Consultation recommendations, and concluded that the single SI decision criterion of SI ≥ 1.8 to classify sensitizers would avoid false negative results as well as indeterminate results, which are not useful for regulatory purposes. Borderline positive results that may occur between 1.8 and 2.5 could be evaluated using other information to confirm the result.

4.1.2 Comments on Revised Draft ICCVAM Recommendations: Test Method Protocol

The Panel concurred with ICCVAM that the validation studies indicated that the standardized protocol was sufficiently transferable and reproducible. The Panel agreed that laboratories should maintain a historical database of positive control SI values and some measure of variability over time. The evaluation of the variation in positive control responses over time has wide applicability to a broad range of test systems.

The Panel agreed with the ICCVAM-recommended protocol, which indicated that all existing toxicological information (e.g., acute toxicity and dermal irritation) and structural and physicochemical information on the test substance of interest (and/or structurally related test substances) should be considered, where available, in selecting three consecutive doses (see **Appendix D2**). The OECD Expert Consultation also agreed and emphasized that the highest dose should be the concentration that maximizes exposure while avoiding systemic toxicity and/or excessive local skin irritation after topical application in the mouse. In the absence of such information, and consistent with the updated ICCVAM-recommended protocol (ICCVAM 2009a), a prescreen test should be performed in order to define the appropriate dose level to test in the LLNA: DA. The Panel and the OECD Expert Consultation agreed in principle with ICCVAM that use of a reduced LLNA: DA test method protocol instead of the multi-dose LLNA: DA test method protocol has the potential to reduce the number of animals used in a test by omitting the middle and low dose groups. However, some members of the OECD Expert Consultation speculated that the reduced LLNA would have limited regulatory use and therefore the extent of potential animal savings is difficult to estimate.

4.1.3 Comments on Revised Draft ICCVAM Recommendations: Future Studies

The Panel concurred with ICCVAM's revised draft recommendations for future studies, emphasizing that additional decision criteria and guidance should be identified for substances that produce SI values in the range of uncertainty, and that the additional decision criteria be reassessed as additional discriminators and data become available (e.g., high-quality human ACD data). While the range of uncertainty is eliminated when using the single decision criterion of SI ≥ 1.8, the OECD Expert Consultation recommended that borderline positive results (i.e., SI values between 1.8 and 2.5) be further evaluated to determine if they are correctly identified as potential skin sensitizers.

The Panel recommended further consideration of statistical issues, including how to determine and evaluate classification methods (i.e., classification cutoff points). The Panel also recommended that future interlaboratory validation studies should simultaneously evaluate intralaboratory reproducibility, using appropriate statistics, to evaluate variation both within a laboratory and between laboratories.

ICCVAM considered the Panel report and the OECD Expert Consultation recommendations and concluded that efforts should be made to further characterize the sensitization potential of borderline positive substances that produce an SI between 1.8 and 2.5 in the LLNA: DA to confirm that such results are not false positive.

4.1.4 Comments on Revised Draft ICCVAM Recommendations: Performance Standards

The Panel agreed that the ICCVAM-recommended LLNA performance standards state the essential test method requirements, and that the LLNA: DA adheres to them such that it should be considered mechanistically and functionally similar. The only variation with the traditional LLNA is the means by which lymphocyte proliferation during the induction phase is evaluated. Likewise, the OECD Expert Consultation also considered the LLNA: DA to be mechanistically and functionally similar to the LLNA, and therefore agreed that the LLNA performance standards are applicable.

4.2 ICCVAM Consideration of Public and SACATM Comments

The ICCVAM evaluation process incorporates a high level of transparency. This process is designed to provide numerous opportunities for stakeholder involvement, including submitting written public comments and providing oral comments at ICCVAM independent peer review panel meetings and SACATM meetings. **Table 4-1** lists the 12 different opportunities for public comment that were provided during the ICCVAM evaluation of the validation status of new versions and applications of the LLNA. The number of public comments received in response to each of the opportunities is also indicated. A total of 49 comments were submitted. Comments received in response to or related to the FR notices are available on the NICEATM-ICCVAM website.[12] The following sections, delineated by FR notice, briefly discuss the public comments received.

Table 4-1 Opportunities for Public Comments

Opportunities for Public Comments	Date	Number of Public Comments Received
72 FR 27815: The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data	May 17, 2007	17
72 FR 52130: Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments	September 12, 2007	4
73 FR 1360: Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments	January 8, 2008	7
Independent Scientific Peer Review Panel Meeting Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay	March 4-6, 2008	16
73 FR 25754: Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)	May 7, 2008	1
73 FR 29136: Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments	May 20, 2008	0

continued

[12] Available at http://ntp-apps.niehs.nih.gov/iccvampb/searchPubCom.cfm

Table 4-1 Opportunities for Public Comments (continued)

Opportunities for Public Comments	Date	Number of Public Comments Received
SACATM Meeting, Radisson Hotel, RTP, NC	June 18-19, 2008	0
74 FR 8974: Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments	February 27, 2009	1
Independent Scientific Peer Review Panel Meeting Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Evaluation of the Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay	April 28-29, 2009	2
74 FR 19562: Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)	April 29, 2009	0
74 FR 26242: Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments	June 1, 2009	1
SACATM Meeting, Hilton Arlington Hotel, Arlington, VA	June 25-26, 2009	0

4.2.1 Public Comments in Response to 72 FR 27815 (May 17, 2007): The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data

NICEATM requested the following:

1. Public comments on the appropriateness and relative priority of evaluation of the validation status of
    a. The LLNA as a stand-alone assay for determining potency (including severity) for the purpose of hazard classification
    b. The reduced LLNA approach (Kimber et al. 2006; ESAC 2007; ICCVAM 2009b)
    c. Nonradioactive LLNA methods
    d. The use of the LLNA for testing mixtures, aqueous solutions, and metals
    e. The current applicability domain
2. Nominations of expert scientists to consider as members of a possible peer review panel
3. Submission of data for the LLNA and/or modified versions of the LLNA

In response to this FR notice, NICEATM received 17 comments. Six comments included additional data and information, while two others offered data and information upon request. Three commenters nominated four potential panelists for consideration. Three commenters suggested reference publications for consideration during the Panel evaluation. The nominees were included in the database of experts from which the Panel was selected. The data and suggested references were included in the ICCVAM draft review documents that were provided to the Panel at the March 2008 meeting.

1. A commenter suggested rearranging the priority sequence of test method evaluation from most to least pressing: a, e, d, b, and c (see list above).

- ICCVAM did not establish a relative priority for these activities because they were all considered to be high-priority activities. Accordingly, all LLNA-related activities described above were discussed at the March 2008 Panel meeting.

One comment pertained to the LLNA: DA.

1. One commenter indicated that several nonradioactive detection methods for the LLNA (e.g., bromodeoxyuridine [BrdU] incorporation, methods measuring the release of various cytokines, methods using fluorescent markers, and quantification by flow cytometry) have been developed and shown to be as sensitive as protocols involving radiolabeling. The commenter indicated that since both ECVAM and JaCVAM were reviewing some of these types of nonradioactive methods that ICCVAM should collaborate with these ongoing efforts rather than initiate a comprehensive independent review.

- In 2007, the CPSC requested that ICCVAM evaluate several modifications of the LLNA, which included the LLNA: DA. After considering comments from the public and the SACATM, ICCVAM assigned the activity a high priority. Scientists from ECVAM and JaCVAM served as liaisons to the IWG during the evaluation of the LLNA: DA and actively participated in the review. Both liaisons nominated scientists to the peer review panel and the JaCVAM liaison provided much of the validation data for the review.

4.2.2 Public Comments in Response to 72 FR 52130 (September 12, 2007): Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments

NICEATM requested public comments on the September 2007 draft ICCVAM-recommended LLNA performance standards developed to facilitate evaluation of modified LLNA test method protocols with regard to the traditional LLNA. In response to this FR notice, NICEATM received four comments, two of which suggested clarifications to the text. Another comment recommended that test substances chosen for testing in the various LLNA methods should be pure, with conclusive structures, and should not be mixtures. Most comments specifically addressed the LLNA performance standards, although one comment pertained to the LLNA in general.

1. One commenter supported the development of performance standards that expedite the validation of new protocols similar to previously validated methods but was disappointed that NICEATM-ICCVAM had chosen to develop performance standards for such a narrow scope of applicability (i.e., modifications of the standard LLNA that involve incorporation of nonradioactive methods of detecting lymphocyte proliferation). The commenter suggested that limited resources available to NICEATM-ICCVAM would be better spent on activities that would have greater impact on the reduction, refinement, or replacement of animal use, such as evaluating the use of human cell lines or *in vitro* skin models as a replacement for the LLNA.

- ICCVAM considered the comment and concluded that the proposed modifications to the LLNA test method protocol and expanded applications have the potential to further reduce and refine animal use. ICCVAM is committed to identifying *in vitro* models and non-animal approaches for assessing ACD and is engaged with ECVAM and JaCVAM in the development of validation studies for such methods.

There were no comments that specifically addressed the LLNA: DA.

4.2.3 Public Comments in Response to 73 FR 1360 (January 8, 2008): Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments

NICEATM requested public comments on the January 2008 draft BRDs, draft ICCVAM test recommendations, draft test method protocols, and revised draft LLNA performance standards for an international independent scientific peer review panel meeting to evaluate modifications and new applications for the LLNA. NICEATM received 23 comments in response to this FR notice; seven written comments were received in advance of the meeting, and 16 oral comments were offered at the Panel meeting.

One written comment was relevant to the LLNA: DA.

1. The commenter indicated that beyond the method to assess lymph node cell proliferation, the test method protocol for the LLNA: DA contained several key deviations from the OECD TG 429 recommended protocol and the essential test method components as described in the January 2008 draft ICCVAM-recommended LLNA performance standards (i.e., major modifications from the traditional LLNA in both the test substance treatment and sampling schedule). The commenter viewed that the LLNA: DA should not be considered for validation as an alternative to the traditional LLNA since the modifications extended beyond the specifications in the January 2008 draft ICCVAM-recommended LLNA performance standards.

- The validation studies for the LLNA: DA test method were completed prior to the development of LLNA performance standards and thus, the ICCVAM-recommended LLNA performance standards were not used to evaluate the LLNA: DA. Further, despite the differences between the LLNA: DA test method protocol and the traditional LLNA test method protocol, ICCVAM concurs with the Panel that the LLNA: DA is mechanistically and functionally similar to the traditional LLNA and therefore the LLNA performance standards would otherwise be applicable.

Two oral comments were relevant to the LLNA: DA.

1. One commenter agreed with ICCVAM that the LLNA: DA (and also the LLNA: BrdU by enzyme-linked immunosorbent assay [ELISA]) should be evaluated separately because of different treatment schedules. The commenter also questioned whether the extra topical dose in the LLNA: DA was necessary, and expressed concern that additional doses may cause skin irritation. For this reason, the commenter suggested that the SI should be evaluated at earlier sample times and without SLS pretreatment.

- Yamashita et al. (2005) examined the effect of various dosing regimens on the SI value produced in the LLNA: DA. The fourth topical application of test substance was required for sensitizers to produce SI $\geq$ 3.0.
- The effect of SLS pretreatment on the SI values of selected substances is presented in the final BRD (**Annex I** of **Appendix C**) and Idehara et al. (2008). Briefly, the data indicated that the calculated EC3 values were lower for substances pretreated with an aqueous solution of 1% SLS than for substances not pretreated with an aqueous solution of 1% SLS. This included some weak sensitizers for which an enhanced response would be important to detect.
- The SLS pretreatment constitutes application of a 1% aqueous solution, which does not induce excessive local skin irritation. SLS is an irritant in mice at 10% in *N,N,*-dimethylformamide (Antonopoulos et al. 2008).

2. Another commenter cited data from Ullmann (2002) that indicates differences in the responsiveness of six different mouse strains (CBA/CaOlaHsd, CBA/Ca [CruBR], CBA/Jlbm [SPF], CBA/JNCrj, BALB/c, and NMRI) to 25% 2-mercaptobenzothiazole. The data showed that CBA/JNCrj mice had markedly lower responses compared to the other strains tested, which may explain the negative result for 2-mercaptobenzothiazole produced by the LLNA: DA test method.

- Validation studies for the LLNA: DA were conducted exclusively with the CBA/JNCrlj strain, which is therefore considered the preferred strain. There were insufficient LLNA: DA data in multiple strains to allow for an evaluation of potential strain differences.

4.2.4 Public Comments in Response to 73 FR 25754 (May 7, 2008): Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

NICEATM announced the SACATM meeting and requested written and public oral comment on the agenda topics. One public comment was received in response to this FR notice. The commenter made a general comment that the members of SACATM do not represent a cross-section of the American public.

- The SACATM charter indicates that the Committee shall consist of 15 members, including the Chair. Voting members shall be appointed by the Director, NIEHS, and include representatives from an academic institution, a State government agency, an international regulatory body, or any corporation developing or marketing new or revised or alternative test methodologies, including contract laboratories. Knowledgeable representatives from public health, environmental communities, or organizations using new or alternative test methodologies may be included as appropriate. There shall be at least one knowledgeable representative having a history of expertise, development, or evaluation of new or revised or alternative test methods from each of the following categories: (1) personal care, pharmaceutical, industrial chemicals, or agricultural industry; (2) any other industry that is regulated by one of the Federal agencies on ICCVAM; and (3) a national animal protection organization established under section 501(c)(3) of the Internal Revenue Code of 1986. The Director, NIEHS, shall select the Chair from among the appointed members of SACATM.

4.2.5 Public Comments in Response to 73 FR 29136 (May 20, 2008): Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

NICEATM requested submission of written public comments on the Independent Scientific Peer Review Panel Assessment. No public comments were received in response to this FR notice.

4.2.6 Public and SACATM Comments: SACATM Meeting on June 18-19, 2008

The June 18-19, 2008, SACATM meeting included a discussion of the ICCVAM review of the LLNA test method (**Appendix F3**).

There were no public comments specific to the LLNA: DA.

Regarding the LLNA: DA, one SACATM member indicated that it was uncertain whether the test method would perform well for mixtures, metals, or aqueous solutions.

- As outlined in the test method recommendations, ICCVAM considers the applicability domain for the LLNA: DA to be the same as the traditional LLNA unless there are properties associated with a class of materials that may interfere with the accuracy of the LLNA: DA. However, inconsistent results for nickel sulfate in the LLNA: DA suggest that the LLNA: DA may not be suitable for testing nickel compounds. Therefore, ICCVAM recommends the accrual of additional data from LLNA: DA studies on such nickel compounds with comparative human and/or guinea pig data in order to more comprehensively evaluate the suitability of the LLNA: DA for testing nickel compounds.

4.2.7 Public Comments in Response to 74 FR 8974 (February 27, 2009): Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments

NICEATM requested public comments on the revised draft BRDs, revised draft ICCVAM test recommendations, and revised draft test method protocols for the second international independent scientific peer review panel meeting to evaluate modifications and new applications for the LLNA. NICEATM received three comments in response to this FR notice: one written comment and two oral comments offered at the Panel meeting.

1. There was a general comment expressing concern that the extensive time and resources that ICCVAM has devoted to this evaluation has detracted from focus on promising *in vitro* methods with potential to have a much greater impact on animal use.

- ICCVAM considers that the evaluations conducted to date have significant potential to further reduce and refine animal use, particularly where the use of the LLNA is precluded due to restrictions associated with the use of radioactivity. ICCVAM is also committed to identifying *in vitro* models and non-animal approaches for assessing ACD and is engaged with ECVAM and JaCVAM in the development of validation studies for such methods.

The commenter further made one written comment relevant to the LLNA: DA.

1. The commenter supported the revised draft ICCVAM recommendation that the LLNA: DA can be used for ACD testing with specific defined limitations in the decision criteria. The commenter viewed that substances falling within the intermediate SI (i.e., when maximum SI results were between the SI decision criteria for sensitizers and nonsensitizers) would be subjected to an integrated decision strategy in conjunction with all other available information (e.g., dose-response information, statistical analyses of treated vs. control animals, peptide reactivity, molecular weight, results from related chemicals, other testing data). While the commenter offered general support for this use, they emphasized that it should be made clear that "other testing data" refers to retrospective analyses rather than initiation of additional tests in animals.

- ICCVAM agrees that additional animal tests should be avoided whenever possible. The intermediate SI range was discarded because it was irrelevant for ICCVAM's final recommendation to use a single decision criterion, SI $\geq$ 1.8, to classify potential sensitizers. However, ICCVAM recommends that borderline positive results (i.e., SI values between 1.8 and 2.5) should be evaluated with other available information (e.g., dose-response information, evidence of systemic toxicity and/or excessive local skin irritation, statistical comparison of treated vs. vehicle control groups [where appropriate], peptide reactivity, molecular weight, results from related substances, other testing data) to confirm that such results are positive.

The commenter further noted that the Panel recommended that the LLNA: DA and the two other nonradioactive methods should be evaluated for their ability to assess mixtures, metals, and aqueous solutions concurrently with the assessment of these substances in the traditional LLNA. The commenter viewed that since the only difference between these methods and the traditional LLNA is the method of detection, it is unlikely that there will be any differences in the applicability of these methods and the traditional LLNA with regard to mixtures, metals, and aqueous solutions. Therefore, it would be highly inappropriate to perform these redundant studies.

- As outlined in the test method recommendations, ICCVAM considers the applicability domain for the LLNA: DA to be the same as the traditional LLNA unless there are properties associated with a class of materials that may interfere with the accuracy of the LLNA: DA. However, inconsistent results for nickel sulfate in the LLNA: DA suggest that the LLNA: DA may not be suitable for testing nickel compounds. Therefore, ICCVAM recommends the accrual of additional data from LLNA: DA studies on such nickel compounds with comparative human and/or guinea pig data in order to more comprehensively evaluate the suitability of the LLNA: DA for testing nickel compounds.

One oral comment was relevant to the LLNA: DA.

1. One commenter stated that the nonradiolabeled LLNA methods should not be held to a higher standard than the traditional LLNA.

- ICCVAM evaluated the LLNA: DA test method based on the applicable criteria for validation and acceptance of toxicological test methods in the ICCVAM submission guidelines (ICCVAM 2003). ICCVAM is committed to ensuring that new methods are equivalent to or better than the currently accepted toxicological methods in order to protect public health.

4.2.8 Public Comments in Response to 74 FR 19562 (April 29, 2009): Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

NICEATM announced the SACATM meeting and requested written and public oral comment on the agenda topics. No public comments were received in response to this FR notice.

4.2.9 Public Comments in Response to 74 FR 26242 (June 1, 2009): Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

NICEATM requested submission of written public comments on the Independent Scientific Peer Review Panel Assessment. One comment was received in response to this FR notice.

The commenter made one comment relevant to the LLNA: DA.

1. The commenter did not consider the nonradioactive LLNA methods to provide significant advantages to the traditional LLNA.

- The ICCVAM recommendations for the nonradioactive test methods state that the proposed nonradioactive modifications to the LLNA test method protocol have significant potential to further reduce and refine animal use, given that they will likely increase the use of the LLNA instead of guinea pig test methods where radioactivity is prohibited.

The commenter also indicated that for the LLNA: DA an explanation of the use of SLS was needed.

- As indicated in Section 2.0 of the final ICCVAM BRD (**Appendix C**), 1% SLS pretreatment is used in the LLNA: DA because various researchers have shown that an aqueous solution of 1% SLS does not elicit a positive response in the traditional LLNA but when applied prior to test substance administration there is generally an increased response compared to the test substance alone (van Och et al. 2000; De Jong et al. 2002).

4.2.10 Public and SACATM Comments: SACATM Meeting on June 25-26, 2009

The June 25-26, 2009, SACATM meeting included a discussion of the ICCVAM review of the LLNA test method (**Appendix F4**).

There were no public comments specific to the LLNA: DA.

In general, SACATM was supportive of the Panel report. However, there was general concern regarding the potential for over-labeling substances that may occur by using LLNA test results. They emphasized the need for developing non-animal test methods for identifying potential skin sensitizers.

Regarding the LLNA: DA, one SACATM member did not consider ATP content to be an accurate measure of lymphocyte proliferation and therefore considered methods that use BrdU incorporation (i.e., LLNA: BrdU-ELISA and LLNA: BrdU by flow cytometry) to be higher priority for moving forward.

- Measuring ATP content by bioluminescence, as is done in the LLNA: DA by the luciferin-luciferase assay, is known to correlate with living cell number (Crouch et al. 1993) and therefore indicates an increased number of proliferating cells in the draining auricular lymph nodes (Ishizaka et al. 1984; Dexter et al. 2003). As indicated in Section 2.0 of the final ICCVAM BRD (**Appendix C**), the emitted light intensity (measured using a luminometer) is linearly related to the ATP concentration and the luciferin-luciferase assay is a sensitive method for ATP quantitation used in a wide variety of applications (Lundin 2000).

Another SACATM member asked if the SLS pretreatment had ever been validated.

- Annex I of the final ICCVAM BRD (**Appendix C**) and Idehara et al. (2008) provide comparative results in the LLNA: DA for a number of substances tested both with and without SLS pretreatment. Briefly, the data indicate that the calculated EC3 values were lower for substances pretreated with SLS than for substances not pretreated with SLS. This included some weak sensitizers for which an enhanced response would be important to detect.

Another SACATM member indicated that the use of two SI decision criteria in the LLNA: DA (i.e., one for determining sensitizers and one for determining nonsensitizers) could potentially place many compounds in the range of uncertainty (i.e., the range in which maximum SI results were between the SI decision criteria for sensitizers and nonsensitizers), so the decision criteria should be reassessed as more data are obtained.

- The final ICCVAM recommendations state that a single decision criterion of SI $\geq$ 1.8 be used to classify substances as potential sensitizers since there were no false negatives in the current validation database, relative to the traditional LLNA, when this criterion is used. However, using an SI $\geq$ 1.8 as the decision criterion results in a false positive rate of 25% (3/12) compared to the traditional LLNA. Since the three false positive substances in the LLNA: DA produced SI values between 1.8 and 2.5, users may want to consider additional information (e.g., dose-response information, evidence of systemic toxicity and/or excessive local skin irritation, statistical comparison of treated vs. vehicle

> control groups [where appropriate], peptide reactivity, molecular weight, results from related substances, other testing data) to confirm that results in this SI range are positive.

Another SACATM member commented that many laboratories had moved away from using the LLNA because it used radioactivity. Therefore, the option of LLNA test method protocols that do not use radioactivity would likely increase use of the LLNA.

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Appendix A

Timeline for ICCVAM Evaluation of the LLNA: DA

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January 10, 2007	ICCVAM receives nomination from CPSC for seven LLNA review activities,[1] including evaluation of the LLNA: DA test method.
January 2007	The ICCVAM IWG is re-established to work with NICEATM to carry out LLNA evaluations.
January 24, 2007	ICCVAM endorses the six CPSC-nominated LLNA review activities and development of ICCVAM LLNA Test Method Performance Standards.
May 17, 2007	*Federal Register* notice (72 FR 27815) – *The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data*.
June 12, 2007	SACATM endorses with high priority the six CPSC-nominated LLNA review activities and development of ICCVAM LLNA Test Method Performance Standards.
September 25–26, 2007	ICCVAM participation in ECVAM Workshop: An Evaluation of Performance Standards and Nonradioactive Endpoints for the Local Lymph Node Assay.
January 8, 2008	*Federal Register* notice (73 FR 1360) – *Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments*.
March 4–6, 2008	Independent Peer Review Panel Meeting on seven LLNA review activities, CPSC Headquarters, Bethesda, MD; public meeting with opportunity for oral public comments.[2]
May 20, 2008	*Federal Register* notice (73 FR 29136) – *Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments*.
June 18–19, 2008	SACATM public meeting for comments on the 2008 Panel report.
February 27, 2009	*Federal Register* notice (74 FR 8974) – *Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments*.
April 28–29, 2009	Independent Peer Review Panel Meeting on LLNA review activities, NIH, Bethesda, MD; public meeting with opportunity for oral public comments.[3]
June 1, 2009	*Federal Register* notice (74 FR 26242) – *Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments*.
June 25–26, 2009	SACATM public meeting for comments on the 2009 Panel report.
October 20–22, 2009	OECD Expert Consultation Meeting, CPSC Headquarters, Bethesda, MD, on proposed updates to TG 429 and two new TG proposals for nonradioactive LLNA test methods (includes the LLNA: DA).

[1] http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf
[2] http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel08.htm
[3] http://iccvam.niehs.nih.gov/methods/immunotox/llna.htm

December 1, 2009	OECD Expert Consultation Teleconference to discuss remaining issues on proposed updates to TG 429 and two new TG proposals for nonradioactive LLNA test methods, which includes the LLNA: DA.
March 23–25, 2010	Meeting of the Working Group of National Co-ordinators of the Test Guidelines Programme to approve adoption of proposed updates to TG 429 and two new TG proposals for nonradioactive LLNA test methods, which includes the LLNA: DA.
March 2010	ICCVAM endorses the TMER for the LLNA: DA, which includes the final background review document.
2010 (published within two weeks after transmittal)	*Federal Register* notice: Announces availability of ICCVAM TMER for the LLNA: DA.

Abbreviations: BRD = background review document; CPSC = U.S. Consumer Product Safety Commission; ECVAM = European Centre for the Validation of Alternative Methods; FR = *Federal Register*; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; IWG = Immunotoxicity Working Group; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; NICEATM = National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods; NIH = National Institutes of Health; OECD = Organisation for Economic Co-operation and Development; SACATM = Scientific Advisory Committee on Alternative Toxicological Methods; TG = Test Guideline; TMER = test method evaluation report.

Appendix B

ICCVAM-Recommended Test Method Protocol: The Murine Local Lymph Node Assay: DA, a Nonradioactive Alternative Test Method to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products

Annex I

An Approach to Dissection and Identification of the Draining ("Auricular") Lymph Nodes B-13

Annex II

Evaluating Local Irritation and Systemic Toxicity in the LLNA: DA B-19

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1.0 General Principle of Detection of Skin Sensitization using the Nonradiolabelled Murine Local Lymph Node Assay: Modified by Daicel Chemical Industries, Ltd., Based on ATP Content (LLNA: DA)

The basic principle underlying the murine local lymph node assay (LLNA) is that sensitizers induce proliferation of lymphocytes in the lymph nodes draining the site of substance application. Under appropriate test conditions, this proliferation is proportional to the dose applied, and provides a means of obtaining an objective, quantitative measurement of sensitization. The test measures cell proliferation as a function of *in vivo* radioisotope (^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine) incorporation into the DNA of dividing lymphocytes, and assesses this proliferation in the draining lymph nodes proximal to the application site (see **Annex I**). Due to the use of radioactivity, the LLNA has limited use in regions where the acquisition, use, or disposal of radioactivity is problematic. The LLNA: DA[1] was therefore developed as a nonradioactive modification to the LLNA that measures increases in ATP content in the lymph node as an indicator of the cell number at the end of cell proliferation (Yamashita et al. 2005; Idehara et al. 2008). The ability to detect skin sensitizers without the necessity of using a radioactive label for DNA eliminates the potential for occupational exposure to radioactivity and waste disposal issues. Similar to the LLNA, the LLNA: DA provides quantitative data suitable for dose-response assessment. The proliferation is proportional to the dose and to the potency of the applied allergen and provides a simple means of obtaining a quantitative measurement of sensitization. The LLNA: DA assesses this proliferation as the proliferation in test groups compared to that in vehicle treated controls. The ratio of the proliferation in treated groups to that in concurrent vehicle treated controls, termed the stimulation index (SI), is determined, and should be ≥1.8 before a test substance can be considered as a skin sensitizer with specific limitations for borderline positive results (i.e., SI between 1.8 and 2.5) as described in Section 3 of this Test Method Evaluation Report.

The methods described here are based on the use of measuring ATP content by luciferin-luciferase assay to indicate an increased number of proliferating cells in the draining auricular lymph nodes. The luciferin-luciferase assay is a sensitive method for ATP quantitation used in a wide variety of applications (Lundin 2000). It utilizes the luciferase enzyme to catalyze the formation of light from ATP and luciferin according to the following reaction:

$$ATP + Luciferin + O_2 \xrightarrow{Luciferase} Oxyluciferin + AMP + PP_i + CO_2 + Light$$

The emitted light intensity is linearly related to the ATP concentration and is measured using a luminometer. A concurrent positive control is added to each assay to provide an indication of appropriate assay performance.

2.0 Description of the LLNA: DA

2.1 Sex and Strain of Animals

The mouse is the species of choice for the LLNA: DA. Validation studies for the LLNA: DA were conducted exclusively with young adult female mice (nulliparous and non-pregnant) of the CBA/JNCrlj strain, and therefore these are the recommended sex and mouse strain.[2] At the start of the

[1] Daicel Chemical Industries, Ltd., Japan.

[2] Male mice and other substrains of CBA mice (e.g., CBA/Ca or CBA/J) may be used if it is sufficiently demonstrated that these animals perform as well as female CBA/JNCrlj mice in the LLNA: DA.

study, mice should be 8-12 weeks of age. All mice should be age matched (preferably within a one-week time frame). Weight variations between the mice should not exceed 20% of the mean weight.

2.2 Preparation of Animals

The temperature of the experimental animal room should be 22°C (±3°C) and the relative humidity 30%-70% (although the aim is for 50%-60%). Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, an unlimited supply of standard laboratory mouse diets and drinking water should be used. The mice should be quarantined/acclimatized for at least five days prior to the start of the test (ILAR 1996). Mice should be allocated to small groups by a stratified randomization or other appropriate methods before the start of the study unless adequate scientific rationale for housing mice individually is provided (ILAR 1996). Four animals per cage is the recommended housing arrangement. The mice are uniquely identified prior to being placed in the study. The method used to mark the mice should not involve identification via the ear (e.g., marking, clipping, or punching of the ear). Colored marks on the tail or other appropriate methods should be used. All mice should be examined (e.g., clinical signs, body weights, observation of excrement) prior to the initiation of the test to ensure good health and the absence of skin lesions.

2.3 Preparation of Doses

Solid test substances should be dissolved or suspended in appropriate solvents/vehicles and diluted, if appropriate, prior to dosing of the mice. Liquid test substances may be dosed directly (i.e., applied neat) or diluted prior to dosing. Insoluble materials, such as those generally seen in medical devices, should be subjected to an exaggerated extraction in an appropriate solvent to reveal all extractable constituents for testing prior to dosing. Fresh preparations of the test substance should be prepared daily unless stability data demonstrate the acceptability of storage.

2.4 Test Conditions

2.4.1 Solvent/vehicle

The solvent/vehicle should not interfere with or bias the test result and should be selected on the basis of maximizing the solubility in order to obtain the highest concentration achievable while producing a solution/suspension suitable for application of the test substance. Recommended vehicles are acetone: olive oil (4:1 v/v), *N,N*-dimethyl-formamide (DMF), methyl ethyl ketone (MEK), propylene glycol, and dimethyl sulfoxide (DMSO) (Van Och et al. 2000; Kimber et al. 1994), but others may be used if sufficient scientific rationale is provided (Kimber and Basketter 1992). Particular care should be taken to ensure that hydrophilic materials are incorporated into a vehicle system that wets the skin and does not immediately run off by incorporation of appropriate solubilizers (e.g., 1% Pluronic® L92). Thus, wholly aqueous vehicles may need to be avoided. In certain situations, it may be necessary for regulatory purposes to test the substance in the clinically relevant solvent or product formulation.

2.4.2 Controls

Concurrent negative (solvent/vehicle) and positive controls should be included in each test to ensure that the test system is functioning properly and that the specific test is valid. In some circumstances (e.g., when using a solvent/vehicle not recommended in **Section 2.4.1**), it may be useful to include a naïve control. Except for treatment with the test substance, the mice in the negative control groups should be handled in an identical manner to the mice of the treatment groups.

Positive controls are used to demonstrate appropriate performance of the assay by responding with adequate and reproducible sensitivity to a sensitizing substance for which the magnitude of the response is well characterized. Inclusion of a concurrent positive control is recommended because it demonstrates competency of the laboratory to successfully conduct each assay and allows for an

assessment of intra- and interlaboratory reproducibility and comparability. The positive control should produce a positive LLNA: DA response resulting in an SI that is at least 1.8 over that observed in the negative control group. The positive control dose should be chosen such that the induction is reproducible but it does not cause excessive skin irritation or systemic toxicity. Preferred positive control substances are 25% hexyl cinnamic aldehyde (HCA; Chemical Abstracts Service Registry Number [CASRN] 101-86-0) or 10% eugenol (CASRN 97-53-0) in acetone: olive oil (4:1 v/v). There may be circumstances in which, given adequate justification, other positive control substances meeting the above criteria may be used.

Although the positive control substance should be tested in the vehicle that is known to elicit a consistent response (e.g., acetone: olive oil), there may be certain regulatory situations in which testing in a nonstandard vehicle (clinically/chemically relevant formulation) will also be necessary. In such situations, the possible interaction of a positive control with this unconventional vehicle should be tested. If the concurrent positive control substance is tested in a different vehicle than the test substance, then a separate vehicle control for the concurrent positive control should be included.

While inclusion of a concurrent positive control group is recommended, there may be situations in which periodic testing (i.e., at intervals ≤6 months) of the positive control substance may be adequate for laboratories that conduct the LLNA: DA regularly (i.e., conduct the LLNA: DA at a frequency of no less than once per month) and have an established historical positive control database that demonstrates the laboratory's ability to obtain reproducible and accurate results with positive controls. Adequate proficiency with the LLNA: DA can be successfully demonstrated by generating consistent results with the positive control in at least 10 independent tests conducted within a reasonable period of time (i.e., less than one year).

A concurrent positive control group should always be included when there is a procedural change to the LLNA: DA (i.e., change in trained personnel, change in test method materials and/or reagents, change in test method equipment, change in source of test animals), and such changes should be documented in laboratory reports. Consideration should be given to the impact of these changes on the adequacy of the previously established historical database in determining the necessity for establishing a new historical database to document consistency in the positive control results.

Investigators should be aware that the decision to conduct a positive control on a periodic basis instead of concurrently has ramifications on the adequacy and acceptability of negative study results generated without a concurrent positive control during the interval between each periodic positive control study. For example, if a false negative result is obtained in the periodic positive control study, all negative test substance results obtained in the interval between the last acceptable periodic positive control study and the unacceptable periodic positive control study may be questioned. Implications of these outcomes should be carefully considered when determining whether to include concurrent positive controls or to only conduct periodic positive controls. Consideration should also be given to using fewer animals in the concurrent positive control group when this is scientifically justified and if the laboratory demonstrates, based on laboratory-specific historical data, that fewer mice can be used without substantially increasing the failure rate of the positive control (i.e., the rate at which SI < 1.8 and the frequency with which studies will need to be repeated due to positive control failure [Appendix A of ICCVAM 2009a]).

In instances where substances of a specific chemical class or range of responses are being evaluated, benchmark substances may be useful to demonstrate that the test method is functioning properly for detecting the skin sensitization potential of a test substance. Appropriate benchmark substances should have the following properties:

- Structural and functional similarity to the class of the substance being tested
- Known physical/chemical characteristics
- Supporting data from the LLNA: DA
- Supporting data on known effects in animal models and/or from humans

2.5 Methodology

A minimum of four animals is used per dose group, with a minimum of three concentrations of the test substance, plus a concurrent negative control group treated only with the vehicle for the test substance, and a concurrent positive control. The processing of lymph nodes from individual mice allows for the assessment of interanimal variability and a statistical comparison of the difference between test substance and vehicle control group measurements. In addition, evaluating the possibility of reducing the number of mice in the positive control group is only feasible when individual animal data are collected.

Test substance treatment dose levels should be based on the recommendations given in Kimber and Basketter (1992) and in the ICCVAM Panel Report (ICCVAM 1999). Consecutive doses are normally selected from an appropriate concentration series such as 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. Adequate scientific rationale should accompany the selection of the concentration series used. All existing toxicological information (e.g., acute toxicity and dermal irritation) and structural and physicochemical information on the test material of interest (and/or structurally related test materials) should be considered, where available, in selecting the three consecutive concentrations so that the highest concentration maximizes exposure while avoiding systemic toxicity and/or excessive local skin irritation (Kimber et al. 1994; OECD 2002). In the absence of such information, an initial prescreen test may be necessary (**Annex II**).

The LLNA: DA experimental procedure is performed as follows:

Day 1. Individually identify and record the weight of each animal and any clinical observations. Apply 1% sodium lauryl sulfate (SLS) aqueous solution to the dorsum of each ear by using a brush dipped in the SLS solution to cover the entire dorsum of each ear with four to five strokes. One hour after the SLS treatment, apply 25 µL of the appropriate dilution of the test substance, the vehicle alone, or the concurrent positive control to the dorsum of each ear.

Days 2, 3, and 7. Repeat the 1% SLS aqueous solution pretreatment and test substance application procedure carried out on Day 1.

Days 4, 5, and 6. No treatment.

Day 8. Record the weight of each animal and any clinical observations. Approximately 24 to 30 hours after the start of application on Day 7, humanely kill the animals. To further monitor the local skin response in the experimental study, additional parameters such as scoring of ear erythema or ear thickness measurements (obtained either by using a thickness gauge, or ear punch weight determinations at necropsy) may be included in the study protocol.

Excise the draining auricular lymph nodes from each mouse ear and process separately in phosphate buffered saline for each animal. Details and diagrams of the node identification and dissection can be found in **Annex I**.

A single-cell suspension of lymph node cells (LNC) excised bilaterally from each mouse is prepared by sandwiching the lymph nodes between two glass slides and applying light pressure to crush the nodes. After confirming that the tissue has spread out thinly pull the two slides apart. Suspend the tissue on both slides in phosphate buffered saline (PBS) by holding each slide at an angle over the petri dish and rinsing with PBS while concurrently scraping the tissue off of the slide with a cell scraper. A total volume of 1 mL PBS should be used for rinsing both slides. The tissue suspension in the petri dish should be homogenized lightly with the cell scraper. A 20 µL aliquot of the

homogenized suspension is then collected with a micropipette and mixed with 1.98 mL PBS to yield a 2 mL sample. This procedure is repeated so that two samples per animal are collected for immediate ATP measurement.

ATP is measured by the luciferin/luciferase method using a commercially available ATP measurement kit that measures bioluminescence in relative luminescence units (RLU). Follow the instructions in the assay kit. The assay timeframe from animal sacrifice to measurement of ATP content for each individual animal should be uniform, within approximately 30 minutes, because the ATP content is considered to gradually decrease with time after animal sacrifice (Idehara et al. 2008). Thus, the series of procedures from excision of auricular lymph nodes to ATP measurement should be completed within 20 minutes by the predetermined time schedule that is the same for each animal. ATP luminescence should be measured in each 2 mL sample so that a total of two ATP measurements are collected for each animal. The mean ATP luminescence is then determined and used in subsequent calculations.

The procedure for preparing the LNC suspension is a critical step of this assay; it is most important to crush the lymph node and suspend the LNC completely. Every technician should establish the skill in advance. The lymph nodes in negative control animals are small, so careful operation is required to avoid an artificial effect on SI values.

2.6 Reduced LLNA

Using this test method protocol, there is also the opportunity to perform a reduced LLNA: DA (rLLNA: DA). Use of the rLLNA: DA has the potential to reduce the number of animals by omitting the middle and low dose groups from the LLNA: DA (Kimber 2006; ESAC 2007; ICCVAM 2009b). This is the only difference between the LLNA: DA and the rLLNA: DA. Thus, the test substance concentration evaluated in the rLLNA: DA should be the maximum concentration that does not induce overt systemic toxicity and/or excessive local irritation in the mouse (**Annex II**). The rLLNA: DA should be used for the hazard classification of skin sensitizing substances if dose-response information is not needed, provided there is adherence to all other LLNA: DA protocol specifications.

2.7 Observations

Mice should be carefully observed at least once daily for any clinical signs, either of local irritation at the application site or of systemic toxicity (**Annex II**). Weighing mice prior to treatment and at the time of necropsy will aid in assessing systemic toxicity. All observations are systematically recorded with records maintained for each individual mouse. Animal monitoring plans should include criteria to promptly identify for euthanasia those mice exhibiting systemic toxicity, excessive irritation, or corrosion of skin (OECD 2000).

3.0 Calculation of Results

Results for each treatment group are expressed as the mean SI. The SI value is derived by dividing the mean RLU/mouse within each test substance group and the concurrent positive control group by the mean RLU/mouse for the solvent/vehicle control group. The average SI value for vehicle treated controls is then one.

The decision process regards a result as positive when SI $\geq$ 1.8 (see Section 3 of this Test Method Evaluation Report). However, the strength of the dose response, chemical toxicity, solubility, and, where appropriate, statistical significance should be considered together with SI values to arrive at a final decision (Basketter et al. 1996; ICCVAM 1999; EPA 1998; Kimber et al. 1998).

Collecting data at the level of the individual mouse will enable a statistical analysis for presence and degree of dose response in the data. Any statistical assessment could include an evaluation of the dose-response relationship as well as suitably adjusted comparisons of test groups (e.g., pairwise

dosed group versus concurrent solvent/vehicle control comparisons). Statistical analyses may include, for instance, linear regression or Williams' test to assess dose-response trends, and Dunnett's test for pairwise comparisons. In choosing an appropriate method of statistical analysis, the investigator should maintain an awareness of possible inequalities of variances and other related problems that may necessitate a data transformation or a nonparametric statistical analysis. In any case, the investigator may need to carry out SI calculations and statistical analyses with and without certain data points (sometimes called "outliers").

4.0 Evaluation and Interpretation of Results

Consideration should be given to the possibility of borderline positive results when SI values between 1.8 and 2.5 are obtained. This is based on the validation database of 44 substances using an SI ≥ 1.8 for which the LLNA: DA correctly identified all 32 LLNA sensitizers, but incorrectly identified three of 12 LLNA nonsensitizers with SI values between 1.8 and 2.5 (i.e. borderline positive) (see Section 3.0 of this Test Method Evaluation Report). If an SI value between 1.8 and 2.5 is obtained, other available information such as dose-response, evidence of systemic toxicity or excessive local skin irritation, and (where appropriate) statistical significance together with SI values should be considered to confirm that such borderline positive results are potential skin sensitizers (see Section 3 of this Test Method Evaluation Report). Consideration should also be given to various properties of the test substance, including whether it has a structural relationship to known skin sensitizers. These and other considerations are discussed in detail elsewhere (Basketter et al. 1998).

Employing the optimized assay condition described previously, the mean SI value for the positive control group (25% HCA or 10% eugenol) should be equal to or greater than 1.8. If not, data derived from the experiment should not be used for evaluation.

5.0 Data and Reporting

5.1 Data

Data should be summarized in tabular form showing the individual animal RLU values, the group mean RLU/animal, its associated error term (e.g., standard deviation [SD], standard error of the mean [SEM]), and the mean SI value for each dose group compared against the concurrent solvent/vehicle control group.

5.2 Test Report

The test report should contain the following information:

Test Substances and Control Substances

- Identification data (e.g. CASRN, if available; source; purity; known impurities; lot number)
- Physical nature and physicochemical properties (e.g. volatility, stability, solubility, physicochemical properties relevant to the conduct of the study)
- Composition and relative percentages of components, if formulation

Solvent/Vehicle

- Identification data (purity; concentration, where appropriate; volume used)
- Justification for choice of vehicle

Test Animals

- Source of CBA mice, housing conditions, diet, etc.
- Microbiological status of the animals, when known
- Number and age of animals

Test Conditions

- Details of test substance preparation and application
- Justification for dose selection (including results from prescreen test, if conducted)
- Vehicle and test substance concentrations used, and total amount of substance applied
- Details of food and water quality (including diet type/source, water source)
- Details of treatment and sampling schedules
- Methods for measurement of toxicity
- Criteria for considering studies as positive or negative
- Details of any protocol deviations and an explanation on how the deviation affects the study design and results

Reliability check

- Summary of results of latest reliability check, including information on substance, concentration and vehicle used
- Concurrent and/or historical positive and negative (solvent/vehicle) control data for testing laboratory
- Date and laboratory report for the most recent periodic positive control and a report detailing the historical positive control data for the laboratory justifying the basis for not conducting a concurrent positive control, if a concurrent positive control was not included

Results

- Individual weights of mice at start of dosing and at scheduled kill; as well as mean and associated error term (e.g., SD, SEM) for each treatment group
- Time course of onset and signs of toxicity, including dermal irritation at site of administration, if any, for each animal
- Table of individual mouse RLU values and SI values for each treatment group
- Mean and associated error term (e.g., SD, SEM) for RLU/mouse for each treatment group and the results of outlier analysis for each treatment group
- Calculated SI and an appropriate measure of variability that takes into account the interanimal variability in both the test substance and control groups
- Dose response relationship
- Statistical analysis, where appropriate

Discussion of the Results

- Brief commentary on the results, the dose-response analysis, and statistical analyses, where appropriate, with a conclusion as to whether the test substance should be considered a skin sensitizer

Conclusion

A Quality Assurance Statement for GLP-compliant Studies

- Indicate all inspections made during the study and the dates any results were reported to the Study Director; confirm that the final report reflects the raw data

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Annex I

An Approach to Dissection and Identification of the Draining ("Auricular") Lymph Nodes

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1.0 Background

Although minimal technical training of the LLNA: DA is required, extreme care must be taken to ensure appropriate and consistent dissection of the lymph nodes. It is recommended that technical proficiency in the dissection and identification of the lymph nodes draining the ear be achieved by practice on mice that have been (a) injected with a colored agent (dye) and/or (b) sensitized with a strong positive sensitizer. Brief descriptions of these practice dissections are provided below. Recognizing that nodes from vehicle-treated and naïve mice are smaller, laboratories performing the LLNA: DA must also gain proficiency in the dissection of these nodes. It may be helpful for laboratories inexperienced in this procedure to request guidance from laboratories that have successfully performed the LLNA: DA.

2.0 Training and Preparation for Node Identification

2.1 Identification of the Draining Node – Dye Treatment

Several methods can be used to provide color identification of the draining nodes. These techniques may be helpful for initial identification and should be performed to ensure proper isolation of the appropriate node. Examples of such treatments are listed below. It should be noted that other such protocols might be used effectively.

Evan's Blue Dye treatment:

Inject approximately 0.1 mL of 2% Evan's Blue Dye (prepared in sterile saline) intradermally into the pinna of an ear. Euthanize the mouse after several minutes and continue with the dissection as noted below.

Colloidal carbon and other dye treatments:

Colloidal carbon and India ink are examples of other dye treatments that may be used (Tilney 1971).

2.2 Identification of the Draining Node – Application of Strong Sensitizers

For the purpose of node identification and training, a strong sensitizer is recommended. This agent should be applied in the standard acetone: olive oil vehicle (4:1). Suggested sensitizers for this training exercise include 0.1% oxazolone, 0.1% (w/v) 2,4-dinitrochlorobenzene, and 0.1% (v/v) dinitrofluorobenzene. After treating the ear with a strong sensitizer, the draining node will dramatically increase in size, thus aiding in identification and location of the node.

Using a procedure similar to that described in the test method protocol, apply the agent to the dorsum of both ears (25 μL/ear) for three consecutive days. On the fourth day, euthanize the mouse. Identification and dissection (listed below) of the node should be performed in these animals prior to practice in non-sensitized or vehicle-treated mice, where the node is significantly smaller.

Please note: Due to the exacerbated response, the suggested sensitizers are not recommended as controls for assay performance. They should only be used for training and node identification purposes.

3.0 Dissection Approach

3.1 Lateral Dissection (Figure B-I-1)

Although lateral dissection is not the conventional approach used to obtain the nodes draining the ear, it may be helpful as a training procedure when used in combination with the ventral dissection. Perform this approach bilaterally (on both sides of the mouse). After euthanizing the mouse, place it in a lateral position. Wet the face and neck with 70% ethanol. Use scissors and forceps to make an initial cut from the neck area slightly below the ear. Carefully extend the incision toward the mouth and nose. Angle the tip of the scissors slightly upward during this procedure to prevent the damage of

deeper tissue. Gently retract the glandular tissue in the area using the forceps. Using the masseter muscle, facial nerves, blood vessels, and the bifurcation of the jugular vein as landmarks, isolate and remove the draining node (**Figure B-I-1**). The draining node ("auricular") will be positioned adjacent to the masseter muscle and proximal to and slightly above the jugular bifurcation.

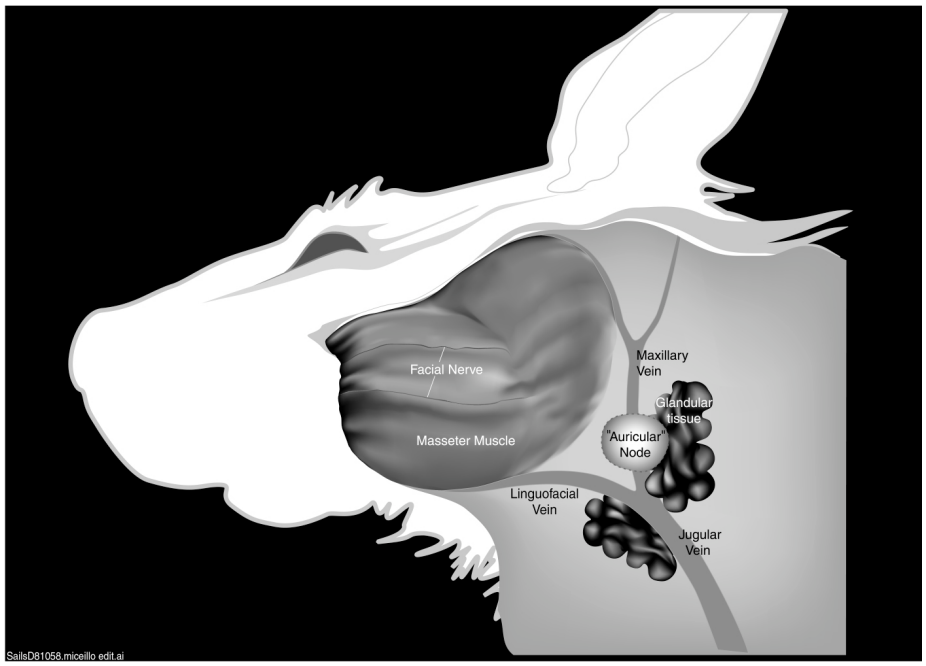
3.2 Ventral Dissection (Figure B-I-2)

The most commonly used dissection approach is from the ventral surface of the mouse. This approach allows both right and left draining nodes to be obtained without repositioning the mouse. With the mouse ventrally exposed, wet the neck and abdomen with 70% ethanol. Use scissors and forceps to carefully make the first incision across the chest and between the arms. Make a second incision up the midline perpendicular to the initial cut, and then cut up to the chin area. Reflect the skin to expose the external jugular veins in the neck area. Take care to avoid salivary tissue at the midline and nodes associated with this tissue. The nodes draining the ear ("auricular") are located distal to the masseter muscle, away from the midline, and near the bifurcation of the jugular veins.

4.0 Accuracy in Identification

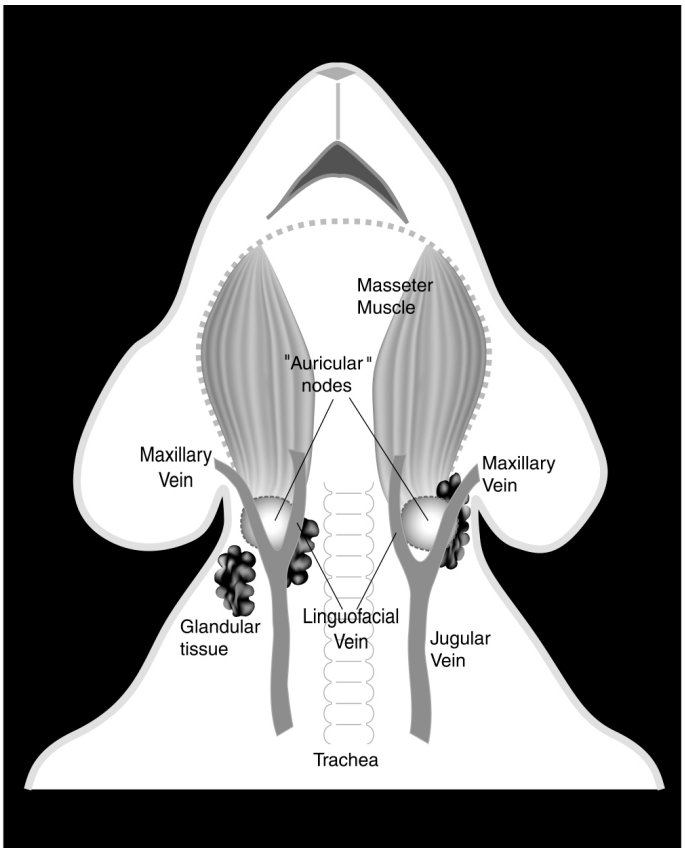
The nodes can be distinguished from glandular and connective tissue in the area by the uniformity of the nodal surface and a shiny translucent appearance. Application of sensitizing agents (especially the strong sensitizers used in training) will cause enlargement of the node size. If a dye is injected for training purposes, the node will take on the tint of the dye.

Figure B-I-1 Lateral Dissection



Credit: Dee Sailstad, U.S. EPA

Figure B-I-2 Ventral Dissection



Credit: Dee Sailstad, U.S. EPA

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Annex II

Evaluating Local Irritation and Systemic Toxicity in the LLNA: DA

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Evaluating Local Irritation and Systemic Toxicity in the LLNA: DA

As noted in the ICCVAM-recommended LLNA: DA test method protocol, the maximum dose tested should be the maximum possible concentration that does not produce systemic toxicity and/or excessive local skin irritation after topical application in the mouse. In the absence of information to determine this concentration (e.g., acute toxicity and dermal irritation data, and/or structural and physicochemical information on the test material and/or structurally related test materials), a prescreen test should be performed using three dose levels of the test substance in order to define the appropriate dose to test in the LLNA: DA.

The prescreen test is conducted under identical conditions as the main LLNA: DA study, except there is no assessment of lymph node proliferation. The maximum dose tested should be 100% of the test material for liquids or the maximum possible concentration for solids or suspensions. One or two animals per dose group are suggested. All mice will be observed daily for any clinical signs of systemic toxicity and/or local skin irritation at the application site. Body weights are recorded pretest and prior to termination (Day 8). Both ears of each mouse are observed for erythema and scored using **Table B-II-1**. Ear thickness measurements are taken using a thickness gauge (e.g., digital micrometer or Peacock Dial thickness gauge) on Day 1 (predose), Day 3 (approximately 48 hours after the first dose), Day 7 (24 hours prior to termination), and Day 8 (termination). Additionally on Day 8, ear thickness could be determined by ear punch weight determinations, which must be performed after the animals are humanely killed. Excessive local irritation is indicated by an erythema score ≥3 and/or an increase in ear thickness of ≥25% on any day of measurement (Reeder et al. 2007; ICCVAM 2009c). The highest dose selected for the main LLNA: DA study will be the next lower dose in the prescreen concentration series that does not induce systemic toxicity and/or excessive local skin irritation.

Table B-II-1 Erythema Scores

Observation	Value
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to eschar formation preventing grading of erythema	4

In addition to a 25% increase in ear thickness (Reeder et al. 2007; ICCVAM 2009c), a statistically significant increase in ear thickness in the treated mice compared to control mice has also been used to identify irritants in the traditional LLNA (Hayes et al. 1998; Homey et al. 1998; Woolhiser et al. 1998; Hayes and Meade 1999; Ehling et al. 2005; Vohr and Jürgen 2005). While statistically significant increases can occur when ear thickness is less than 25%, they have not been associated specifically with excessive irritation (Woolhiser et al. 1998; Hayes and Meade 1999; Ehling et al. 2005; Vohr and Jürgen 2005; Patterson et al. 2007).

Test guidelines for assessing acute dermal toxicity recommend a number of clinical observations for assessing systemic toxicity (OECD 1987; EPA 1998). The following clinical observations, which are based on test guidelines and current practices (ICCVAM 2009d), may indicate systemic toxicity when used as part of an integrated assessment and therefore may indicate the maximum dose level to use in the main LLNA: DA:

- Changes in nervous system function (e.g., piloerection, ataxia, tremors, and convulsions)
- Changes in behavior (e.g., aggressiveness, change in grooming activity, marked change in activity level)
- Changes in respiratory patterns (i.e., changes in frequency and intensity of breathing such as dyspnea, gasping, and rales)
- Changes in food and water consumption
- Lethargy and/or unresponsiveness
- Any clinical signs of more than slight or momentary pain and distress
- Reduction in body weight >5% from Day 1 to Day 8
- Mortality

Moribund animals or animals showing signs of severe pain and distress should be humanely killed (OECD 2000).

Appendix C

Final Background Review Document:
The Nonradioactive Murine Local Lymph Node Assay: DA

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Background Review Document
Nonradioactive Murine Local Lymph Node Assay: DA

Interagency Coordinating Committee on the
Validation of Alternative Methods

National Toxicology Program Interagency Center for the
Evaluation of Alternative Toxicological Methods

National Institute of Environmental Health Sciences
National Institutes of Health
U.S. Public Health Service
Department of Health and Human Services

March 2010

National Toxicology Program
P.O. Box 12233
Research Triangle Park, NC 27709

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List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
ACE	Acetone
Anim.	Animal
ANOVA	Analysis of variance
AOO	Acetone: olive oil (4:1)
aq.	Aqueous
BRD	Background review document
Calc.	Calculated
CASRN	Chemical Abstracts Service Registry Number
CPSC	U.S. Consumer Product Safety Commission
CI	Confidence interval
Conc.	Concentration
CV	Coefficient of variation
Cys	Cysteine-containing peptide
DMF	*N,N*-dimethylformamide
DMSO	Dimethyl sulfoxide
EC1.8	Estimated concentration needed to produce a stimulation index of 1.8
EC2	Estimated concentration needed to produce a stimulation index of two
EC2.5	Estimated concentration needed to produce a stimulation index of 2.5
EC3	Estimated concentration needed to produce a stimulation index of three
ECt	Estimated concentration needed to produce a stimulation index of a specified threshold
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ECVAM	European Centre for the Validation of Alternative Methods
EPA	U.S. Environmental Protection Agency
FN	False negative
FP	False positive
GP	Guinea pig
GPMT	Guinea pig maximization test
HMT	Human maximization test
HPTA	Human patch test antigen
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IDR	Insufficient dose response
ILS	Integrated Laboratory Systems
ISO	International Organization for Standardization
IWG	Immunotoxicity Working Group
JaCVAM	Japanese Center for the Validation of Alternative Methods

K_{ow}	Estimated log octanol-water partition coefficient
LLNA	Murine local lymph node assay
LLNA: DA	Murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content
LLNA: BrdU-ELISA	Murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine
MEK	Methyl ethyl ketone
MHLW	Ministry of Health, Labour and Welfare (Japan)
Min	Minimal
Mod	Moderate
Mol.	Molecular
NA	Not applicable
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NR	Not reported
NT	Not tested
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate buffered saline
PC	Positive control
Ref.	Reference
rLLNA: DA	Reduced murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content
RLU	Relative luminescence units
SD	Standard deviation
SI	Stimulation index
SLS	Sodium lauryl sulfate
Stats.	Statistics
TG	Test guideline
Trad.	Traditional
U.S.	United States
Unk	Unknown
VC	Vehicle control
Veh.	Vehicle
vs.	Versus

Interagency Coordinating Committee on the Validation of Alternative Methods: Agency Representatives

Agency for Toxic Substances and Disease Registry
* Moiz Mumtaz, Ph.D.
Bruce Fowler, Ph.D.
Edward Murray, Ph.D.
Eric Sampson, Ph.D.

Consumer Product Safety Commission
* Marilyn L. Wind, Ph.D. (Chair)
+ Kristina Hatlelid, Ph.D.
Joanna Matheson, Ph.D.

Department of Agriculture
* Jodie Kulpa-Eddy, D.V.M. (Vice-Chair)
+ Elizabeth Goldentyer, D.V.M.

Department of Defense
* Robert E. Foster, Ph.D.
+ Patty Decot
Harry Salem, Ph.D.
Peter J. Schultheiss, D.V.M., DACLAM

Department of Energy
* Michael Kuperberg, Ph.D.
+ Marvin Stodolsky, Ph.D.

Department of the Interior
* Barnett A. Rattner, Ph.D.
+ Sarah Gerould, Ph.D. (to Feb. 2009)

Department of Transportation
* George Cushmac, Ph.D.
+ Steve Hwang, Ph.D.

Environmental Protection Agency
Office of Pesticide Programs
* John R. "Jack" Fowle III, Ph.D., DABT
+ Vicki Dellarco, Ph.D.
+ Tina Levine, Ph.D.
Deborah McCall
Christine Augustyniak, Ph.D. (*U.S. Coordinator, OECD Test Guidelines Program)*
Office of Pollution Prevention and Toxics
Jerry Smrchek, Ph.D. (*U.S. Coordinator, OECD Test Guidelines Program,* to July 2009*)*
Office of Research and Development
Suzanne McMaster, Ph.D. (to Dec. 2008)
Julian Preston, Ph.D. (to July 2009)
Stephanie Padilla, Ph.D. (to July 2009)
Office of Science Coordination and Policy
Karen Hamernik, Ph.D. (to July 2009)

Food and Drug Administration
Office of the Commissioner
* Suzanne Fitzpatrick, Ph.D., DABT
Center for Biologics Evaluation and Research
Richard McFarland, Ph.D., M.D.
Ying Huang, Ph.D.
Center for Devices and Radiological Health
Melvin E. Stratmeyer, Ph.D.
Vasant G. Malshet, Ph.D., DABT
Center for Drug Evaluation and Research
+ Abigail C. Jacobs, Ph.D.
Paul C. Brown, Ph.D.
Center for Food Safety and Applied Nutrition
David G. Hattan, Ph.D.
Robert L. Bronaugh, Ph.D.
Center for Veterinary Medicine
Devaraya Jagannath, Ph.D.
M. Cecilia Aguila, D.V.M.
National Center for Toxicological Research
Paul Howard, Ph.D.
Donna Mendrick, Ph.D.
William T. Allaben, Ph.D. (to Jan. 2009)
Office of Regulatory Affairs
Lawrence D'Hoostelaere, Ph.D.

National Cancer Institute
* T. Kevin Howcroft, Ph.D.
Chand Khanna, D.V.M., Ph.D.
Alan Poland, M.D. (to Oct. 2008)

National Institute of Environmental Health Sciences
* William S. Stokes, D.V.M., DACLAM
+ Raymond R. Tice, Ph.D.
Rajendra S. Chhabra, Ph.D., DABT
Jerrold J. Heindel, Ph.D.

National Institute for Occupational Safety and Health
* Paul Nicolaysen, V.M.D.
+ K. Murali Rao, M.D., Ph.D.

National Institutes of Health
* Margaret D. Snyder, Ph.D.

National Library of Medicine
* Pertti (Bert) Hakkinen, Ph.D.
+ Jeanne Goshorn, M.S.

Occupational Safety and Health Administration
* Surender Ahir, Ph.D.

* Principal agency representative
+ Alternate principal agency representative

Acknowledgements

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Immunotoxicity Working Group (IWG)

U.S. Consumer Product Safety Commission
Joanna Matheson, Ph.D. (IWG Co-chair)
Marilyn L. Wind, Ph.D.

U.S. Environmental Protection Agency
Office of Pesticide Programs
Jonathan Chen, Ph.D.
Masih Hashim, D.V.M., Ph.D.
Marianne Lewis
Deborah McCall
Timothy McMahon, Ph.D.
John Redden, M.S.
Jenny Tao, Ph.D.
Office of Pollution Prevention and Toxics
Elizabeth Margosches, Ph.D.
Ronald Ward, Ph.D.
Office of Research and Development
Marsha Ward, Ph.D.
Office of Science Coordination and Policy
Karen Hamernik, Ph.D.

U.S. Food and Drug Administration
Center for Devices and Radiological Health
Vasant G. Malshet, Ph.D., DABT
Jeffrey Toy, Ph.D.
Center for Drug Evaluation and Research
Ruth Barratt, Ph.D., D.V.M.
Paul C. Brown, Ph.D.
Abigail C. Jacobs, Ph.D. (IWG Co-chair)
Jiaqin Yao, Ph.D.
Office of Science and Health Coordination
Suzanne Fitzpatrick, Ph.D., DABT

National Institute of Environmental Health Sciences
Dori Germolec, Ph.D.
William S. Stokes, D.V.M., DACLAM

National Institute for Occupational Safety and Health
B. Jean Meade, D.V.M., Ph.D.

National Library of Medicine
Pertti Hakkinen, Ph.D.

European Centre for the Validation of Alternative Methods – Liaison
Silvia Casati, Ph.D.
Alexandre Angers, Ph.D.

Japanese Center for the Validation of Alternative Methods – Liaison
Hajime Kojima, Ph.D.

National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

National Institute of Environmental Health Sciences

William Stokes, D.V.M., DACLAM
Director; Project Officer

Deborah McCarley
Special Assistant; Assistant Project Officer

NICEATM Support Contract Staff (Integrated Laboratory Systems [ILS], Inc.)

David Allen, Ph.D.
Thomas Burns, M.S.
Linda Litchfield
Steven Morefield, M.D.
Michael Paris
Eleni Salicru, Ph.D.
Catherine Sprankle
Frank Stack
Judy Strickland, Ph.D., DABT
Linda Wilson

Statistical Consultant for ILS, Inc.

Joseph Haseman, Ph.D.

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Daicel Chemical Industries, Ltd.
Hyogo, Japan

Takashi Omori, Ph.D.
Kyoto University School of Public Health
Kyoto, Japan

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Preface

In 1999, the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended the murine (mouse) local lymph node assay (LLNA) as a valid test method to assess the skin sensitization potential of most types of substances (ICCVAM 1999; Sailstad et al. 2001; Dean et al. 2001; Haneke et al. 2001). ICCVAM concluded that the LLNA (referred to herein as the "traditional LLNA") provided several advantages compared to guinea pig test methods, including elimination of potential pain and distress, use of fewer animals, less time required to perform, and availability of dose-response information. United States and international regulatory authorities subsequently accepted the traditional LLNA as an alternative test method for allergic contact dermatitis testing. It is now commonly used around the world.

One disadvantage of the traditional LLNA is that it requires injection of a radioactive marker to measure cell proliferation in lymph nodes. To avoid the use of radioactive markers, scientists have recently developed several nonradioactive versions of the LLNA. In 2007, the U.S. Consumer Product Safety Commission (CPSC) asked ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to evaluate the scientific validity of these nonradioactive versions. ICCVAM assigned the nomination a high priority, and established the ICCVAM Immunotoxicity Working Group (IWG) to work with NICEATM to review the current literature and evaluate available data to assess the validity of three such test methods. The evaluation process involved two public meetings of an international independent scientific peer review panel (referred to hereafter as "Panel") that reviewed draft and revised draft background review documents and ICCVAM test method recommendations.

A comprehensive draft background review document (BRD) provided the initial information, data, and analyses supporting the validation status of each of the nonradioactive test methods. ICCVAM also developed draft test method recommendations for each test method regarding its usefulness and limitations, test method protocol, performance standards, and future studies. NICEATM and ICCVAM provided the draft BRDs and draft test method recommendations to the Panel for their consideration at a public meeting on March 4-6, 2008. A report of the Panel meeting was subsequently published on the NICEATM-ICCVAM website.[1] Both the Panel and ICCVAM concluded that more information was needed before a recommendation on the usefulness and limitations of each of the three test methods could be made. The Panel recommended that NICEATM obtain additional existing data that were not available to the Panel and reanalyze the performance of each nonradioactive LLNA test method. NICEATM subsequently obtained additional data and prepared revised draft BRDs. ICCVAM also prepared revised draft test method recommendations based on the revised draft BRDs. NICEATM and ICCVAM provided the revised draft BRDs and revised draft test method recommendations to the Panel for their consideration at a public meeting on April 28-29, 2009. A report of the Panel meeting was subsequently published on the NICEATM-ICCVAM website.[2]

Based on the revised draft ICCVAM recommendations, NICEATM submitted a proposed draft Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) for the LLNA modified by Daicel Chemical Industries, Ltd., based on ATP content (referred to hereafter as the "LLNA: DA") that was circulated in July 2009 to the 30 OECD member countries for review and comment. An OECD Expert Consultation Meeting was held on October 20-22, 2009, to evaluate the comments. The expert group reviewed the draft OECD TG for the LLNA: DA and proposed responses to the comments from member countries. A revised TG was again distributed to the 30 OECD member countries in December 2009 for review and comment and then the final draft was

[1] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf.
[2] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2009.pdf.

forwarded to the OECD Working Group of National Co-ordinators of the Test Guidelines Programme to consider for adoption at their March 23-25, 2010, meeting.

ICCVAM considered the conclusions and recommendations of the Panel and conclusions from the OECD Expert Consultation, along with comments received from the public and the Scientific Advisory Committee on Alternative Toxicological Methods (the ICCVAM-NICEATM advisory committee), and then finalized the BRDs and test method recommendations. These will be forwarded to Federal agencies for their consideration and acceptance decisions, where appropriate. This BRD addresses the validation database for the LLNA: DA.

We gratefully acknowledge the organizations and scientists who provided data and information for this document. We would also like to recognize the efforts of the individuals who contributed to its preparation, review, and revision. We especially recognize the Panel members for their thoughtful evaluations and generous contributions of time and effort. Special thanks are extended to Dr. Michael Luster for serving as the Panel Chair and to Dr. Michael Woolhiser, Dr. Michael Olson, Kim Headrick, and Dr. Stephen Ullrich for their service as Evaluation Group Chairs. We thank Drs. Abigail Jacobs (U.S. Food and Drug Administration) and Joanna Matheson (CPSC) for serving as Co-chairs of the IWG, as well as the members of the IWG and ICCVAM representatives who subsequently reviewed and provided comments throughout the process leading to this final BRD.

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Marilyn Wind, Ph.D.
Deputy Associate Executive Director
Directorate for Health Sciences
CPSC
Chair, ICCVAM

RADM William S. Stokes, D.V.M., DACLAM
Assistant Surgeon General, U.S. Public Heath Service
Director, NICEATM
Executive Director, ICCVAM

March 2010

Executive Summary

Background

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended to U.S. Federal agencies that the murine local lymph node assay (LLNA) is a valid substitute for currently accepted guinea pig (GP) test methods to assess the allergic contact dermatitis (ACD) potential of many, but not all, types of substances. ACD is an allergic skin reaction characterized by redness, swelling, and itching that can result from contact with a sensitizing chemical or product. The recommendation was based on a comprehensive evaluation that included an international independent scientific peer review panel (Panel) assessment of the validation status of the LLNA. The Panel report and the ICCVAM recommendations (ICCVAM 1999) are available at the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)-ICCVAM website.[3] The LLNA was subsequently incorporated into national and international test guidelines for the assessment of skin sensitization (Organisation for Economic Co-operation and Development [OECD] Test Guideline 429 [OECD 2002]; International Organization for Standardization [ISO] 10993-10: Tests for Irritation and Delayed-type Hypersensitivity [ISO 2002]; U.S. Environmental Protection Agency [EPA] Health Effects Test Guidelines on Skin Sensitization [EPA 2003]).

In 2007, the U.S. Consumer Product Safety Commission (CPSC) formally nominated several activities related to the LLNA for evaluation by ICCVAM and NICEATM.[4] One of the nominated activities was an assessment of the validation status of nonradioactive modifications to the current version of the LLNA ([ICCVAM 1999; Sailstad et al. 2001; Dean et al. 2001; Haneke et al. 2001] referred to hereafter as the "traditional LLNA"), which uses radioactivity to detect sensitizers. The information described in this background review document (BRD) was compiled by ICCVAM and NICEATM in response to this nomination. The BRD provides a comprehensive review of data and information regarding the usefulness and limitations of one of these test methods, the LLNA modified by Daicel Chemical Industries, Ltd., based on ATP content in the draining auricular lymph nodes (referred to hereafter as the "LLNA: DA").

Test Method Protocol

Daicel Chemical Industries, Ltd. developed the LLNA: DA test method based on modifications to the traditional LLNA (Yamashita et al. 2005). While the traditional LLNA assesses cell proliferation by measuring the incorporation of radioactivity into the DNA of dividing lymph node cells, the LLNA: DA assesses cell proliferation by measuring increases in ATP content in the lymph node as an indicator of the cell number at the end of cell proliferation. The LLNA: DA also differs from the traditional LLNA in the timing and administration of the test substance. In the traditional LLNA, the test substance is applied on days 1, 2, and 3 and the auricular lymph nodes are excised on day 6. In the LLNA: DA, the test substance is applied on days 1, 2, 3, and 7 and the auricular lymph nodes are excised on day 8. Furthermore, one hour prior to each application of the test substance, 1% aqueous solution of sodium lauryl sulfate is applied to increase absorption of the test substance through the skin. A stimulation index (SI) is used to identify a substance as a sensitizer (the ratio of the mean ATP content of the substance treatment group to the mean ATP content of the vehicle treatment group).

Validation Database

The accuracy and reliability of the LLNA: DA were assessed using data submitted to NICEATM for 45 substances tested in one laboratory (Idehara et al. 2008; Idehara unpublished) and 14 substances

[3] http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf.
[4] http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf.

tested in a two-phased interlaboratory validation study (17 laboratories) (Omori et al. 2008). Of the 14 substances tested in the two-phased interlaboratory study (Omori et al. 2008) only one was different from the 45 substances tested initially (Idehara et al. 2008; Idehara unpublished). Thus, data were available for 46 unique substances tested in the LLNA: DA. The reference test data for these substances were obtained from the traditional LLNA, GP skin sensitization tests, and/or human skin sensitization tests. One substance, benzocaine, yielded both positive and negative results in the traditional LLNA (ICCVAM 1999) and therefore was not considered in the performance evaluation of the LLNA: DA. LLNA studies for another substance, toluene 2,4-diisocyanate (van Och et al. 2000), were not conducted according to the traditional LLNA test method protocol described (ICCVAM 1999; Dean et al. 2001). Thus of the 46 substances with LLNA: DA data, 44 substances had adequate traditional LLNA data (32 were classified by the traditional LLNA as skin sensitizers and 12 were classified as nonsensitizers).

Test Method Accuracy

The accuracy evaluation in this BRD includes the evaluation of multiple decision criteria, including the SI ≥ 3.0 recommended by the test method developer. Based on the evaluation of multiple decision criteria, the optimal performance was achieved using SI ≥ 1.8 to classify potential skin sensitizers. Compared to the traditional LLNA, accuracy was 93% (41/44), with a false positive rate of 25% (3/12), and a false negative rate of 0% (0/32). The three false positive substances produced SI values between 1.8 and 2.5 in the LLNA: DA.

When the decision criterion of SI ≥ 3.0 was used to classify sensitizers versus nonsensitizers, compared to the traditional LLNA, accuracy was 91% (40/44), with a false positive rate of 0% (0/12), and a false negative rate of 13% (4/32). Among the four discordant substances, no unique characteristics were identified that could be used as rationale for excluding any particular types of substances from testing in the LLNA: DA.

The reduced LLNA: DA (rLLNA: DA), which uses only the highest dose of the test substance that does not elicit excessive skin irritation and/or systemic toxicity, has the potential to reduce animal use by up to 40% for hazard classification purposes when dose-response information is not needed. Using SI ≥ 1.8 to classify potential sensitizers for 123 individual tests which used multiple doses, overall accuracy of the rLLNA: DA compared to the multi-dose LLNA: DA was 98% (121/123), with a false positive rate of 0% (0/33) and a false negative rate of 2% (2/90). The two tests that were false negative in the rLLNA: DA were borderline positive in the LLNA: DA at a concentration lower than the highest dose (maximum SI = 1.97 and 2.00). The highest dose tested for each of the two tests of the two substances was 50%.

Test Method Reliability – Intralaboratory Reproducibility

Intralaboratory reproducibility for the LLNA: DA was assessed using data for two substances (isoeugenol and eugenol) that were tested at varying concentrations in three different experiments. The coefficient of variation (CV) for the reproducibility of the EC3 values (estimated concentration needed to produce an SI of three) for isoeugenol and eugenol was 21% and 11%, respectively. The CV for the reproducibility of the EC1.8 values (estimated concentration needed to produce an SI of 1.8) for isoeugenol and eugenol was 36% and 23%, respectively.

Test Method Reliability – Interlaboratory Reproducibility

This BRD includes a reproducibility analysis using SI ≥ 1.8 to identify potential sensitizers. The two-phased multilaboratory validation study included 17 different laboratories in which 14 different substances were examined. In the first phase of the study, 10 laboratories each tested up to 12 substances, while in the second phase of the study seven laboratories (different from the 10 laboratories in the first phase of the interlaboratory validation study) each tested up to five substances (2/5 substances unique compared to the first phase). In both studies, each substance was tested once at

three different doses, which were provided to the participating laboratories by the validation study management team.

When using SI ≥ 1.8 as the decision criterion, the qualitative (positive/negative) interlaboratory concordance analysis for the 12 substances that were tested in up to 10 laboratories during the first phase of the LLNA: DA interlaboratory validation study resulted in 100% (3/3 or 10/10) concordance for 9 substances (seven sensitizers and two nonsensitizers in the traditional LLNA), 90% (9/10) concordance for one substance (one nonsensitizer in the traditional LLNA), and 67% (2/3) concordance for two substances (two sensitizers in the traditional LLNA). The coefficient of variation (CV) values for the estimated concentration needed to produce a stimulation index of 1.8 (EC1.8) values ranged from 15% (abietic acid) to 140% (isoeugenol) and the mean CV was 71%. The qualitative interlaboratory concordance analysis for the five substances tested in up to seven laboratories during the second phase of the validation study resulted in 100% (4/4 or 7/7) concordance for four substances (three sensitizers and one nonsensitizer in the traditional LLNA) and 75% (3/4) concordance for one substance (a sensitizer in the traditional LLNA). The CV values for the EC1.8 values ranged from 14% (hexyl cinnamic aldehyde) to 93% (cobalt chloride) and the mean CV was 49%.

When using SI ≥ 1.8 to classify potential sensitizers, the tally of concordant tests for the 14 substances with multiple LLNA: DA tests indicated that the SI results for 80% (8/10) of the sensitizers (based on traditional LLNA results) were 100% concordant in the LLNA: DA (i.e., all tests for that substance yielded maximum SI ≥ 1.8). The concordance of the other two sensitizers (based on traditional LLNA results) was 50% (4/8) to 67% (2/3) for SI ≥ 1.8. The SI results for 75% (3/4) of the nonsensitizers (based on traditional LLNA results) were 100% concordant in the LLNA: DA (i.e., all tests for that substance yielded maximum SI ≤ 1.8). The concordance of the other nonsensitizer (based on traditional LLNA results) was 91% (10/11) for SI ≤ 1.8.

Animal Welfare Considerations

The LLNA: DA will use the same number of animals when compared to the updated ICCVAM-recommended LLNA protocol (ICCVAM 2009). However, since use of the traditional LLNA is restricted in some institutions because it involves radioactivity, availability and use of the nonradioactive LLNA: DA may lead to further reduction in use of the GP tests, which would provide for reduced animal use and increased refinement due to the avoidance of pain and distress in the LLNA procedure.

Further, the LLNA: DA evaluates the induction phase of sensitization and therefore discomfort to animals associated with the elicitation phase is eliminated. Additionally, the LLNA: DA protocol requires fewer mice per treatment group (a minimum of four animals per group) than either of the guinea pig tests (10-20 animals/group for the Buehler test and 5-10 animals/group for the guinea pig maximization test [GPMT]).

Test Method Transferability

The transferability of the LLNA: DA was demonstrated by a two-phased interlaboratory validation study (Omori et al. 2008). Notably, the test method developer indicates that when the LLNA: DA test method is conducted, all the procedural steps from lymph node excision to the determination of ATP content should be performed without delay since ATP content decreases over time (Idehara et al. 2008; Omori et al. 2008). Compared to the traditional LLNA, the LLNA: DA will not require facilities, equipment, and licensing permits for handling radioactive materials. The level of training and expertise needed to conduct the LLNA: DA should be similar to the traditional LLNA except that the understanding and practice of luciferase methodology is required.

1.0 Introduction

1.1 Public Health Perspective

Allergic contact dermatitis (ACD) is a frequent occupational health problem that often results in lost workdays[5] and can significantly diminish quality of life (Hutchings et al. 2001; Skoet et al. 2003). ACD develops in two phases, induction and elicitation. The induction phase occurs when a susceptible individual is exposed topically to a skin-sensitizing substance. Induction depends on the substance passing through the epidermis, where it forms a hapten complex with dermal proteins. The Langerhans cells, the resident antigen-presenting cells in the skin, process the hapten complex. The processed hapten complex then migrates to the draining lymph nodes. Antigen presentation to T-lymphocytes follows, which leads to the clonal expansion of these cells. At this point, the individual is sensitized to the substance (Basketter et al. 2003; Jowsey et al. 2006). Studies have shown that the magnitude of lymphocyte proliferation correlates with the extent to which sensitization develops (Kimber and Dearman 1991, 1996).

The elicitation phase occurs when the individual is again topically exposed to the same substance. As in the induction phase, the substance penetrates the epidermis, is processed by the Langerhans cells, and presented to circulating T-lymphocytes. The antigen-specific T-lymphocytes are then activated, which causes release of cytokines and other inflammatory mediators. This release produces a rapid dermal immune response that can lead to ACD (ICCVAM 1999; Sailstad et al. 2001; Basketter et al. 2003; Jowsey et al. 2006).

1.2 Historical Background for the Murine Local Lymph Node Assay

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended that the murine local lymph node assay (LLNA) is a valid substitute for currently accepted guinea pig (GP) test methods to assess the ACD potential of many, but not all, types of substances. The recommendation was based on a comprehensive evaluation that included an independent scientific peer review panel (Panel) assessment of the validation status of the LLNA. The Panel report and the ICCVAM recommendations (ICCVAM 1999) are available at the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)-ICCVAM website.[6] ICCVAM forwarded recommendations to U.S. Federal agencies that the LLNA should be considered for regulatory acceptance or other nonregulatory applications for assessing the ACD potential of substances, while recognizing that some testing situations would still require the use of traditional GP test methods (ICCVAM 1999; Sailstad et al. 2001). The LLNA was subsequently incorporated into national and international test guidelines for the assessment of skin sensitization (Organisation for Economic Co-operation and Development [OECD] Test Guideline [TG] 429 [OECD 2002]; International Standards Organization [ISO] 10993-10: Tests for Irritation and Delayed-type Hypersensitivity [ISO 2002]; U.S. Environmental Protection Agency [EPA] Health Effects Test Guidelines on Skin Sensitization [EPA 2003]).

On January 10, 2007, the U.S. Consumer Product Safety Commission (CPSC) formally nominated several activities related to the LLNA for evaluation by ICCVAM and NICEATM.[7] One of the nominated activities was an assessment of the validation status of nonradioactive modifications to the current version of the LLNA ([ICCVAM 1999; Dean et al. 2001] referred to hereafter as the "traditional LLNA"), which uses radioactivity to detect sensitizers. The information described in this background review document (BRD) was compiled by ICCVAM and NICEATM in response to this nomination. This BRD provides a comprehensive review of available data and information regarding the usefulness and limitations of one of these test methods, the LLNA modified by Daicel Chemical

[5] http://www.bls.gov/IIF
[6] http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf.
[7] http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf.

Industries, Ltd., based on ATP content (referred to hereafter as the "LLNA: DA") in the draining auricular lymph nodes. ICCVAM and its Immunotoxicity Working Group (IWG) evaluated this method in a draft BRD and developed draft test method recommendations based on this initial evaluation.

A Panel reviewed the draft BRD in March 2008 to evaluate the extent to which the information contained in the draft BRD supported the draft test method recommendations. The Panel concluded that additional information was needed to evaluate the test method, including a detailed test method protocol, quantitative data for the test method, and an evaluation of interlaboratory reproducibility. In response to this recommendation, NICEATM obtained additional LLNA: DA data and information, which were used to generate a revised draft BRD for review by the Panel in April 2009.

Based on the revised draft ICCVAM test method recommendations, NICEATM submitted a proposed draft OECD TG for the LLNA: DA that was circulated in July 2009 to the 30 OECD member countries for review and comment via their National Co-ordinators, who distributed the draft TG to interested stakeholders. An OECD Expert Consultation meeting was held on October 20-22, 2009, to evaluate the comments. Scientists from the National Institute of Environmental Health Sciences, the Environmental Protection Agency, the Food and Drug Administration, and CPSC, as well as U.S. and international experts from industry and other stakeholder organizations, participated in this meeting, which was co-hosted by CPSC and NICEATM-ICCVAM. The expert group reviewed the draft OECD TG for the LLNA: DA and proposed responses to comments from member countries. The OECD Expert Consultation convened a subsequent teleconference on December 1, 2009, to discuss outstanding issues identified at the October meeting. A revised TG was distributed to the 30 OECD member countries in December 2009, via their National Co-ordinators, for review and comment by national experts and interested stakeholders. A final teleconference of the OECD Expert Consultation was convened on January 29, 2010 to discuss the member country comments received during the last round of review, and a final draft TG was developed based on these discussions. This final draft was forwarded to the OECD Working Group of National Co-ordinators of the Test Guidelines Programme to consider for adoption at their March 23-25, 2010, meeting.

ICCVAM and the IWG considered the conclusions and recommendations of the Panel, comments received from the public and its advisory committee (the Scientific Advisory Committee on Alternative Toxicological Methods), along with the conclusions of the OECD Expert Consultation on the LLNA, and developed this final BRD. ICCVAM provides this final BRD to regulatory agencies for consideration as part of the ICCVAM Test Method Evaluation Report.

1.3 The LLNA: DA

Daicel Chemical Industries, Ltd. developed the LLNA: DA as a nonradioactive modification (Yamashita et al. 2005; Idehara et al. 2008) to the traditional LLNA. The traditional LLNA assesses cell proliferation by measuring the incorporation of radioactive thymidine or iodine into the DNA of dividing lymph node cells. In contrast, the LLNA: DA assesses increases in ATP content in the draining auricular lymph nodes by employing a luciferin-luciferase assay to measure bioluminescence. Since ATP content is linearly related to living cell number, this measurement serves as a surrogate for cell number at the time of sampling (Crouch et al. 1993).

This document provides:

- A comprehensive summary of the LLNA: DA test method protocol
- The substances used in the validation of the test method and the test results
- The performance characteristics (accuracy and reliability) of the test method
- Animal welfare considerations
- Other considerations relevant to the usefulness and limitations of this test method (e.g., transferability, cost of the test method)

2.0 LLNA: DA Test Method Protocol

This BRD includes the detailed standard operating procedure for the LLNA: DA test method that was used in the validation studies (**Annex I**). The LLNA: DA test method protocol (**Annex I**) differs from the ICCVAM-recommended test method protocol for the traditional LLNA (ICCVAM 2009) in the method used to assess lymphocyte proliferation in the auricular lymph nodes (**Table C-1**). In addition, there are substantive differences between the two test method protocols regarding test substance application and timing for the collection of the lymph nodes. In the traditional LLNA, the test substance is administered on three consecutive days (days 1, 2, and 3). On day 6, radiolabeled thymidine or iodine is administered via the tail vein and the lymph nodes are excised five hours later. A lymph node cell suspension is then prepared and radioactive thymidine or iodine incorporation is determined by β-scintillation or γ-scintillation counting, respectively. In the LLNA: DA, the test substance is applied on days 1, 2, 3, and additionally on day 7. During the initial development of the LLNA: DA, the study group (Yamashita et al. 2005) determined the optimal dosing schedule by evaluating whether the addition of a fourth application (day 7) was useful for increasing lymph node proliferation. Based on a statistically significant increase in lymph node weight-based stimulation index (SI) values for mice that received a fourth application (day 7) of the test substance, this test method protocol was chosen. Furthermore, one hour prior to each application of the test substance, an aqueous solution of 1% sodium lauryl sulfate (SLS) is applied to the dorsum of the treated ears to increase absorption of the test substance across the skin (van Och et al. 2000). Various researchers have shown that an aqueous solution of 1% SLS does not elicit a positive response in the traditional LLNA but when applied prior to test substance administration there is generally an increased response compared to the test substance alone (van Och et al. 2000; De Jong et al. 2002). Idehara et al. (2008) observed similar results (see also **Annex I** for supplemental data submitted to NICEATM evaluating the effect of 1% SLS pretreatment on lymph node cell proliferation [Idehara unpublished]). Lastly, 24 to 30 hours after the last test substance application on day 7, the auricular lymph nodes are excised and a lymph node cell suspension is prepared, and the ATP content is measured by luciferin-luciferase assay (day 8). The luciferin-luciferase assay is a sensitive method for ATP quantitation used in a wide variety of applications (Lundin 2000). It utilizes the luciferase enzyme to catalyze the formation of light from ATP and luciferin according to the following reaction:

$$ATP + Luciferin + O_2 \xrightarrow{Luciferase} Oxyluciferin + AMP + PP_i + CO_2 + Light$$

The emitted light intensity is linearly related to the ATP concentration and is measured using a luminometer.

Table C-1 Comparison of the LLNA: DA and Traditional LLNA Experimental Procedure

Day	LLNA: DA	Traditional LLNA
1, 2, & 3	• Pretreat with 1% SLS aqueous solution • After one hour, apply 25 μL of test substance or vehicle to dorsum of each ear	• Apply 25 μL of test substance or vehicle to dorsum of each ear
4 & 5	• No treatment	• No treatment
6	• No treatment	• Administer ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine via tail vein • Excision of auricular lymph nodes • Measurement of radioactivity incorporated into lymph node cells
7	• Pretreat with 1% SLS aqueous solution • After one hour, apply 25 μL of test substance or vehicle to dorsum of each ear	• No treatment
8	• Excision of auricular lymph nodes • Measurement of ATP content in lymph node cells	• No treatment

Abbreviations: ^{3}H = tritiated; ^{125}I = iodine-125; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SLS = sodium lauryl sulfate.

2.1 Decision Criteria

Similar to the traditional LLNA, an SI is used in the LLNA: DA to distinguish skin sensitizers from nonsensitizers. The formula for calculating the SI in the LLNA: DA is the ratio of the mean ATP content of the auricular lymph nodes collected from the test substance treatment group to the mean ATP content of the auricular lymph nodes collected from the vehicle treatment group (measured in relative luminescence units; RLU):

$$SI = \frac{mean\ ATP\ content\ of\ auricular\ lymph\ nodes\ in\ test\ treatment\ group\ (RLU)}{mean\ ATP\ content\ of\ auricular\ lymph\ nodes\ in\ vehicle\ treatment\ group\ (RLU)}$$

In the intra- and interlaboratory validation studies for the LLNA: DA, an SI ≥ 3.0 was used as the threshold for identifying a substance as a sensitizer, which is the same threshold used in the traditional LLNA. As noted in **Section 6.0**, alternative decision criteria are evaluated in this BRD to determine the threshold that provides optimum performance.

3.0 LLNA: DA Validation Database

To evaluate the usefulness and limitations of the LLNA: DA, Daicel Chemical Industries, Ltd. tested a total of 45 substances in one laboratory (Idehara et al. 2008; Idehara unpublished). They further evaluated two of the 45 substances (isoeugenol and eugenol) in the LLNA: DA at varying concentrations in three different experiments in order to assess intralaboratory reproducibility. In addition, a two-phased interlaboratory validation study evaluated the reproducibility of the LLNA: DA (**Section 7.0**). In the first phase 10 laboratories tested 12 coded substances and in the second phase seven different laboratories tested five coded substances. Between the 17 laboratories, 14 different substances were examined and one of those substances, 3-aminophenol, was not previously tested among the 45 substances in the intralaboratory validation study, yielding a total of 46 substances tested in the LLNA: DA.

All 46 substances tested in the LLNA: DA were previously tested in the traditional LLNA, including 40 substances that were considered in the original ICCVAM evaluation of the traditional LLNA (ICCVAM 1999). Cinnamic alcohol, diethyl maleate, ethyl acrylate, glutaraldehyde, methyl methacrylate, and toluene 2,4-diisocyanate were the six substances tested in the LLNA: DA not evaluated in the ICCVAM 1999 report.

Of the 46 substances tested in the LLNA: DA, 33 were classified by the LLNA as skin sensitizers,[8] 12 were classified as nonsensitizers, and one (benzocaine) was classified as equivocal due to highly variable results and therefore was not included in the performance analyses (ICCVAM 1999)[9] (**Table C-2**). For the sensitizers in the LLNA, the range of traditional LLNA EC3 values (estimated concentrations needed to produce an SI of three) was from 0.009% to 90% (**Table C-2**). Similar to benzocaine, LLNA data for toluene 2,4-diisocyanate, not evaluated in the original ICCVAM 1999 report, were not suitable for comparison. The LLNA test method protocol followed for the study that tested toluene 2,4-diisocyanate (van Och et al. 2000) was a modified version of the traditional LLNA which was not performed in accordance with OECD TG 429 (OECD 2002) or ICCVAM 1999 and Dean et al. (2001). One variation included use of the BALB/c strain of mouse for the experiments, and not the CBA/Ca or CBA/J strains as specified by ICCVAM (1999), Dean et al. (2001) or OECD TG 429 (2002). In addition, the ears of the mice were pretreated with an aqueous solution of 1% SLS before treatment with the test substance. The authors also stated that the auricular lymph nodes were excised and pooled for each animal. Thus, of the 46 substances with LLNA: DA and LLNA data, 44 had adequate traditional LLNA data and were included in the accuracy analyses described in **Section 6.0**.

Annex II provides information on physicochemical properties (e.g., physical form tested). For the 44 substances that were evaluated in the LLNA: DA performance analyses, the molecular weights ranged from 30 to 388 g/mol. Twenty-two of the 44 substances were solids, 21 were liquids, and one substance (benzalkonium chloride) exists as either a solid or a liquid. The estimated log octanol-water partition coefficients (K_{ow}) were available for 38 substances and ranged from -8.28 to 6.46. Peptide reactivity, which was available for 28 substances, ranged from high to minimal (Gerberick et al. 2004, 2007).

Annex II further provides information on the Chemical Abstracts Service Registry Number (CASRN) and chemical class for each substance tested. When available, chemical classes for each substance were retrieved from the National Library of Medicine Medical Subject Headings. If

[8] Resorcinol was classified as a nonsensitizer based on original LLNA data (ICCVAM 1999) but recent LLNA data have instead suggested that it is actually a sensitizer (Basketter et al. 2007a) and is therefore classified as a sensitizer for this evaluation.

[9] A series of 12 tests conducted in two laboratories resulted in some positive results that were not reproducible (Basketter et al. 1995).

chemical classes were not located, they were assigned for each test substance using a standard classification scheme, based on the National Library of Medicine Medical Subject Headings classification system.[10] A substance could be assigned to more than one chemical class; however, no substance was assigned to more than three classes. Classification of substances into chemical classes is not intended to indicate the impact of structure on biological activity with respect to sensitization potential. Instead, chemical class information is being presented to provide an indication of the variety of structural elements that are present in the substances that were evaluated in this analysis.

Table C-2 shows that 20 chemical classes are represented by the 44 substances tested in the LLNA: DA with adequate traditional LLNA data; 13 substances were classified in more than one chemical class. The classes with the highest number of substances were carboxylic acids (16 substances) and phenols (five substances). Further, of the 22 chemical classes represented in the NICEATM LLNA database by at least five substances (thereby providing a sufficiently large representation for further analyses), 20 classes had at least 60% of the traditional LLNA results identified as positive. For this database of more than 600 substances, these classes were identified as those most likely to be associated with skin sensitization. Seventeen of these classes were also represented in the LLNA: DA database (only amides, ketones, and macromolecular substances were not included). Among the chemical classes that have been previously identified as common skin allergens (e.g., aldehydes, ketones, quinones, and acrylates, [Gerberick et al. 2004]), only ketones were not included in the LLNA: DA database.

[10] http://www.nlm.nih.gov/mesh/meshhome.html.

Table C-2 Product Use, Chemical Classification, and Traditional LLNA EC3 Values of 46 Substances Tested in the LLNA: DA

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (%) (Max. SI)[3]	N[4]
5-Chloro-2-methyl-4-isothiazolin-3-one[5]	Cosmetics; Manufacturing; Pesticides	Sulfur Compounds; Heterocyclic Compounds	0.009 (27.7)	1
p-Benzoquinone[5]	Manufacturing; Pesticides; Pharmaceuticals	Quinones	0.010 (52.3)	1
2,4-Dinitrochlorobenzene[6, 7]	Manufacturing; Pesticides	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated; Nitro Compounds	0.049 (43.9)	15
Benzalkonium chloride[6]	Cosmetics; Disinfectant; Manufacturing; Personal care products; Pesticides	Amines; Onium Compounds	0.070[8] (11.1)	1
Glutaraldehyde[6, 7]	Cosmetics; Disinfectant; Manufacturing; Pesticides	Aldehydes	0.083 (18.0)	3
p-Phenylenediamine[6]	Intermediate in chemical synthesis; Manufacturing	Amines	0.110 (26.4)	6
Toluene 2,4-diisocyanate[6, 9]	Intermediate in chemical synthesis	Hydrocarbons, Cyclic; Isocyanates	0.110 (NR)	1
Potassium dichromate[6, 10]	Manufacturing; Pharmaceuticals	Inorganic Chemical, Chromium Compounds; Inorganic Chemical, Potassium Compounds	0.170 (33.6)	12
Propyl gallate[5]	Cosmetics; Food additive	Carboxylic Acids	0.320 (33.6)	1
Phthalic anhydride[6]	Intermediate in chemical synthesis; Manufacturing; Pharmaceuticals	Anhydrides; Carboxylic Acids	0.360 (26.0)	1
Formaldehyde[6, 7]	Disinfectant; Manufacturing	Aldehydes	0.495 (4.0)	4
Cobalt chloride[6, 7, 10]	Manufacturing; Pesticides	Inorganic Chemical, Elements; Inorganic Chemical, Metals	0.600 (7.2)	2

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (%) (Max. SI)[3]	N[4]
Isoeugenol[6, 7]	Food additive; Fragrance agent	Carboxylic Acids	1.540 (31.0)	47
2-Mercaptobenzothiazole[6]	Manufacturing; Pesticides	Heterocyclic Compounds	1.700 (8.6)	1
Cinnamic aldehyde[6]	Cosmetics; Food additive; Fragrance agent; Intermediate in chemical synthesis; Personal care products; Pesticides	Aldehydes	1.910 (18.4)	6
3-Aminophenol[7]	Cosmetics; Pharmaceuticals	Amines; Phenols	3.200 (5.7)	1
Benzocaine[6]	Medication	Carboxylic Acids	3.400[11] (7.6)	1
Diethyl maleate[5]	Food additive; Intermediate in chemical synthesis	Carboxylic Acids	3.600 (22.6)	4
Trimellitic anhydride[6]	Manufacturing	Anhydride; Carboxylic Acids	4.710 (4.6)	2
Nickel (II) sulfate hexahydrate[6, 7, 10]	Manufacturing	Inorganic Chemical, Elements; Inorganic Chemical, Metals	4.800 (3.1)	1
Resorcinol[6]	Cosmetics; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Phenols	6.330 (10.4)	1
Sodium lauryl sulfate[6]	Cosmetics; Food additive; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Alcohols; Sulfur Compounds; Lipids	8.080 (8.9)	5
Citral[6]	Fragrance agent	Hydrocarbons, Other	9.170 (20.5)	6
Hexyl cinnamic aldehyde[6, 7, 10]	Food additive; Fragrance agent	Aldehydes	9.740 (20.0)	21

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (%) (Max. SI)[3]	N[4]
Eugenol[6]	Cosmetics; Food additive; Intermediate in chemical synthesis; Manufacturing; Personal care products; Pharmaceuticals	Carboxylic Acids	10.090 (17.0)	11
Abietic acid[6, 7]	Manufacturing	Hydrocarbons, Cyclic; Polycyclic Compounds	11.920 (5.2)	5
Phenyl benzoate[5]	Manufacturing; Pesticides	Carboxylic Acids	13.600 (11.1)	3
Cinnamic alcohol[5]	Cosmetics; Food additive; Fragrance agent; Intermediate in chemical synthesis; Personal care products	Alcohols	21.000 (5.7)	1
Hydroxycitronellal[6]	Food additive; Fragrance agent; Personal care products	Hydrocarbons, Other	23.750 (8.5)	6
Imidazolidinyl urea[6]	Cosmetics; Personal care products; Pesticides	Urea	24.000 (5.5)	1
Ethylene glycol dimethacrylate[5]	Manufacturing	Carboxylic Acids	28.000 (7.0)	1
Butyl glycidyl ether[5]	Intermediate in chemical synthesis; Manufacturing	Ethers	30.900 (5.6)	1
Ethyl acrylate[5]	Manufacturing	Carboxylic Acids	32.800 (4.0)	2
Methyl methacrylate[5]	Manufacturing	Carboxylic Acids	90.000 (3.6)	1
1-Bromobutane[6]	Intermediate in chemical synthesis; Pharmaceuticals; Solvent	Hydrocarbons, Halogenated	NA (1.2)	1
Chlorobenzene[6]	Manufacturing; Solvent	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated	NA (1.7)	1

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (%) (Max. SI)[3]	N[4]
Diethyl phthalate[6]	Cosmetics; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Carboxylic Acids	NA (1.5)	1
Dimethyl isophthalate[5, 7]	Manufacturing; Fragrance agent	Carboxylic Acids	NA (1.0)	1
Hexane[6]	Manufacturing; Solvent	Hydrocarbons, Acyclic	NA (2.2)	1
Isopropanol[6, 7]	Cosmetics; Disinfectant; Food additive; Intermediate in chemical synthesis; Manufacturing; Personal care products; Pharmaceuticals; Solvent	Alcohols	NA (1.7)	1
Lactic acid[6, 10]	Food additive; Manufacturing; Pharmaceuticals	Carboxylic Acids	NA (2.2)	1
Methyl salicylate[6, 7]	Cosmetics; Food additive; Fragrance agent; Personal care products; Pharmaceuticals; Solvent	Carboxylic Acids; Phenols	NA (2.9)	9
Propylparaben[6]	Food additive; Pesticides; Pharmaceuticals	Carboxylic Acids; Phenols	NA (1.4)	1
Nickel (II) chloride[5]	Manufacturing; Pesticides	Inorganic Chemical, Elements; Inorganic Chemical, Metals	NA (2.4)	2
Salicylic acid[5]	Food additive; Manufacturing; Pharmaceuticals	Phenols; Carboxylic Acids	NA (2.5)	1
Sulfanilamide[5]	Pharmaceuticals	Hydrocarbons, Cyclic; Sulfur Compounds	NA (1.0)	1

Abbreviations: EC3 = estimated concentration needed to produce a stimulation index of three; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; Max. = maximum; NA = not applicable; NR = not reported; SI = stimulation index.

[1] Information for product use was gathered from the following databases:
Hazardous Substances Database (HSDB)-National Library of Medicine-TOXNET http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB
Haz-Map: National Library of Medicine-Toxicology and Environmental Health Information Program http://hazmap.nlm.nih.gov/
Household Products Database-National Library of Medicine http://hpd.nlm.nih.gov/index.htm
International Programme on Chemical Safety (IPCS) INCHEM database in partnership with Canadian Centre for Occupational Health and Safety (CCOHS) http://www.inchem.org/
National Toxicology Program http://ntp.niehs.nih.gov:8080/index.html?col=010stat

[2] Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, as developed by the National Library of Medicine: http://www.nlm.nih.gov/mesh/meshhome.html.

[3] The traditional LLNA EC3 value (estimated concentration needed to produce a stimulation index of three) listed for each substance is averaged from respective studies. The substance was tested in the same vehicle in both the traditional LLNA and the LLNA: DA (**Annex IV)**, except where noted. Numbers in parentheses indicate the maximum stimulation index, where reported.

[4] Number of traditional LLNA studies from which the data were obtained.

[5] Substance tested in intralaboratory validation study (Idehara unpublished).

[6] Substance tested in intralaboratory validation study (Idehara et al. 2008).

[7] Substance tested in first phase of a two-phased interlaboratory validation study (Omori et al. 2008).

[8] Benzalkonium chloride was tested in the LLNA: DA using acetone: olive oil (4:1) as the vehicle (**Annex IV**) but the traditional LLNA EC3 value reported is based on results using acetone as the vehicle.

[9] Not included in accuracy analyses. Comparable LLNA reference data from modified LLNA test (van Och et al. 2000).

[10] Substance tested in second phase of a two-phased interlaboratory validation study (Omori et al. 2008).

[11] Not included in accuracy analyses. EC3 value reported in **Table C-2** for benzocaine is based on data from the NICEATM database but variable and equivocal (i.e., results that were not reproducible) responses were reported by Basketter et al. (1995) and in the 1999 ICCVAM report.

4.0 Reference Data

As mentioned in **Section 3.0**, 44 of the 46 substances tested in the LLNA: DA have adequate traditional LLNA data and are included in the accuracy analyses described in **Section 6.0.** The traditional LLNA reference data used for the accuracy analyses comparisons are from ICCVAM (1999) (**Annex III**) for 34 of those 44 substances. The traditional LLNA reference data for the remaining 10 substances (benzalkonium chloride, cinnamic alcohol, diethyl maleate, diethyl phthalate, ethyl acrylate, formaldehyde, glutaraldehyde, imidazolidinyl urea, methyl methacrylate, and nickel [II] sulfate hexahydrate) were obtained from other sources (**Annex III**) (Gerberick et al. 1992; Hilton et al. 1998; Ryan et al. 2002; Basketter et al. 2005; Gerberick et al. 2005; Betts et al. 2006). In addition, Basketter et al. (2007a) reassessed the skin sensitization potential of resorcinol in the LLNA, in accordance with OECD TG 429 (2002), which updates information in the ICCVAM 1999 report and from Gerberick et al. (2005) that had previously stated that this substance tested negative in the LLNA.

The reference data for the GP tests (guinea pig maximization test or Buehler test) and human tests (human maximization test, human patch test allergen, or other human data) were obtained from Vandenberg and Epstein (1963), Kligman (1966a, 1966b, 1966c), Marzulli and Maibach (1974), Jordan and King (1977), Klecak et al. (1977), Marzulli and Maibach (1980), Van der Walle et al. (1982), Gad et al. (1986), Robinson et al. (1990), Gerberick et al. (1992), ICCVAM (1999), Basketter et al. (1999a, 1999b, 2001, 2005, 2007a), Kwon et al. (2003), Schneider and Akkan (2004), and Betts et al. (2006).

An independent quality assurance contractor for the National Toxicology Program audited the traditional LLNA data provided in the ICCVAM 1999 report. Audit procedures and findings are presented in the quality assurance report on file at the National Institute of Environmental Health Sciences. The audit supports the conclusion that the transcribed test data in the submission were accurate, consistent, and complete as compared to the original study records.

5.0 LLNA: DA Test Method Data and Results

The test method data in this BRD include the individual animal data for the LLNA: DA results from the validation studies by Idehara et al. (2008) and Omori et al. (2008). In addition, individual animal data for 14 unpublished studies (Idehara unpublished) were submitted to NICEATM and were included in the evaluation (although the individual animal data were submitted to NICEATM they are not included in the BRD at the request of the test method developer since they are not yet published). **Annex III** represents a summary of data for the 46 different substances tested in the LLNA: DA, and includes the comparative traditional LLNA data that were available for 44 of the 46 substances (see also **Section 3.0**). In addition, 42 of the 46 substances examined in the LLNA: DA have GP data and 43 of the 46 substances tested have human skin sensitization data. Based on Idehara et al. (2008; unpublished), the 45 substances tested in the intralaboratory study were not coded prior to testing. However, the two-phased interlaboratory validation study used coded substances (Omori et al. 2008). Original data for these studies are included in **Annex IV**.

6.0 LLNA: DA Test Method Accuracy

A critical component of a formal evaluation of the validation status of a test method is an assessment of the accuracy of the proposed test method when compared to the current reference test method (ICCVAM 2003). Additional comparisons should also be made against any available human data or experience from testing or accidental exposures. This aspect of assay performance is typically evaluated by calculating:

- *Accuracy (concordance)*: the proportion of correct outcomes (positive and negative) of a test method
- *Sensitivity*: the proportion of all positive substances that are classified as positive
- *Specificity*: the proportion of all negative substances that are classified as negative
- *False positive rate*: the proportion of all negative substances that are incorrectly identified as positive
- *False negative rate*: the proportion of all positive substances that are incorrectly identified as negative

6.1 LLNA: DA Database Used for the Accuracy Analysis

An accuracy analysis for the LLNA: DA test method was conducted using data from the intralaboratory validation study (Idehara et al. 2008; Idehara unpublished) and the two-phased interlaboratory validation study (Omori et al. 2008). Taken together, LLNA: DA test data were available for 46 different substances, 44 of which had adequate comparative traditional LLNA data to conduct an accuracy analysis (**Section 3.0**). Thus, of the 44 substances included in the accuracy analysis, 40 had LLNA: DA, traditional LLNA, and GP data and 41 had LLNA: DA, traditional LLNA, and human data. Classification of substances and data available for each substance are provided in **Annex III**.

Multiple LLNA: DA tests were available for 14 substances tested in the intralaboratory (Idehara et al. 2008; Idehara unpublished) and the two-phased interlaboratory LLNA: DA studies (Omori et al. 2008). For the accuracy analyses, the test results were combined so that each substance was represented by one overall result for the SI analyzed and represented the outcome that was most prevalent. For example, when using SI ≥ 3.0 as the decision criterion, cobalt chloride was positive because five of the eight LLNA: DA results were positive (**Annex IV**). Also, using SI ≥ 3.0 as the decision criterion, inconsistent test results were noted for two of the 14 substances with multiple test results: cobalt chloride and nickel (II) sulfate hexahydrate. Three of the validation laboratories that tested cobalt chloride reported SI < 3.0 and five laboratories yielded SI ≥ 3.0. For nickel (II) sulfate hexahydrate, six validation laboratories reported SI < 3.0 and two laboratories yielded SI ≥ 3.0.

6.2 Accuracy Analysis Using the SI ≥ 3.0 Decision Criterion

The performance characteristics of the LLNA: DA test method were first evaluated using the decision criterion of SI ≥ 3.0 to identify sensitizers, which was the threshold for a positive response used in both the intralaboratory and two-phased interlaboratory validation studies (**Annex I**).

6.2.1 Accuracy vs. the Traditional LLNA

Based on the data (44 substances), when compared to the traditional LLNA, the LLNA: DA had an accuracy of 91% (40/44), a sensitivity of 88% (28/32), a specificity of 100% (12/12), a false positive rate of 0% (0/12), and a false negative rate of 13% (4/32) (**Table C-3**).

6.2.2 Accuracy vs. Guinea Pig Data

When the accuracy statistics for the LLNA: DA and the traditional LLNA were compared for substances with LLNA: DA, traditional LLNA, and GP data, and GP results served as the reference data, the LLNA: DA had a lower accuracy (78% [31/40] vs. 85% [34/40]), sensitivity (85% [22/26]

vs. 96% [25/26]), the same specificity (64% [9/14]) and false positive rate (36% [5/14]), and higher false negative rate (15% [4/26] vs. 4% [1/26]) relative to the traditional LLNA (**Table C-3**).

6.2.3 Accuracy vs. Human Data

When substances with only comparative LLNA: DA, traditional LLNA, and human data were evaluated, and human outcomes served as the reference point, the LLNA: DA had lower accuracy (76% [31/41] vs. 85% [35/41]) and sensitivity (74% [26/35] vs. 86% [30/35]), the same specificity (83% [5/6]) and false positive rate (17% [1/6]), and higher false negative rate (26% [9/35] vs. 14% [5/35]) relative to the traditional LLNA (**Table C-3**).

Table C-3 Performance of the LLNA: DA in Predicting Skin Sensitization Potential Using Decision Criterion of SI ≥ 3.0 to Identify Sensitizers

Comparison	n[1]	Accuracy % (No.[2])	Sensitivity % (No.[2])	Specificity % (No.[2])	False Positive Rate % (No.[2])	False Negative Rate % (No.[2])	Positive Predictivity % (No.[2])	Negative Predictivity % (No.[2])
LLNA: DA vs. Traditional LLNA	44	91 (40/44)	88 (28/32)	100 (12/12)	0 (0/12)	13 (4/32	100 (28/28)	75 (12/16)
Substances with LLNA: DA, Traditional LLNA, and GP Data								
LLNA: DA vs. Traditional LLNA	40	93 (37/40)	90 (27/30)	100 (10/10)	0 (0/10)	10 (3/30)	100 (27/27)	77 (10/13)
LLNA: DA vs. GP[3]	40	78 (31/40)	85 (22/26)	64 (9/14)	36 (5/14)	15 (4/26)	81 (22/27)	69 (9/13)
Traditional LLNA vs. GP[3]	40	85 (34/40)	96 (25/26)	64 (9/14)	36 (5/14)	4 (1/26)	83 (25/30)	90 (9/10)
Substances with LLNA: DA, Traditional LLNA, and Human Data								
LLNA: DA vs. Traditional LLNA	41	90 (37/41)	87 (27/31)	100 (10/10)	0 (0/10)	13 (4/31)	100 (27/27)	71 (10/14)
LLNA: DA vs. Human[4]	41	76 (31/41)	74 (26/35)	83 (5/6)	17 (1/6)	26 (9/35)	96 (26/27)	36 (5/14)
Traditional LLNA vs. Human[4]	41	85 (35/41)	86 (30/35)	83 (5/6)	17 (1/6)	14 (5/35)	97 (30/31)	50 (5/10)

Abbreviations: GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; No. = number; SI = stimulation index; vs. = versus.

[1] n = Number of substances included in this analysis.

[2] The proportion on which the percentage calculation is based.

[3] GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.

[4] Human refers to outcomes obtained by studies conducted using the human maximization test, inclusion of the test substance in a human patch test allergen kit, and/or published clinical case studies/reports.

6.3 Accuracy Analysis (SI ≥ 3.0) Based on ICCVAM-recommended LLNA Performance Standards Reference Substances

In conjunction with the European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM), ICCVAM has developed internationally harmonized test method performance standards for the traditional LLNA (ICCVAM 2009),[11] which are proposed to evaluate the performance of modified LLNA test methods that are mechanistically and functionally similar to the traditional LLNA. Since the validation studies for the LLNA: DA test method were completed prior to the development of LLNA performance standards, the LLNA: DA is not being evaluated using the ICCVAM-recommended LLNA performance standards. Thus, evaluations of the LLNA: DA test substances to the ICCVAM-recommended LLNA performance standards test substances are shown to provide a general comparison to a set list of reference substances (18 required reference substances and four optional reference substances) that represent a diverse substance group.

As shown in **Table C-4**, all of the 18 required reference substances and three of the four optional reference substances included in the ICCVAM-recommended LLNA performance standards have been tested in the LLNA: DA. When compared to the traditional LLNA, the LLNA: DA at SI ≥ 3.0 (SI decision criterion used in the intralaboratory and the interlaboratory validation studies) predicted the same sensitization classification for 16 of the 18 required ICCVAM-recommended reference substances tested. One discordant substance, 2-mercaptobenzothiazole, was classified as a sensitizer based on traditional LLNA results (EC3 = 1.7%) but as a nonsensitizer based on LLNA: DA data. As indicated in **Table C-4**, *N,N*-dimethylformamide (DMF) was the vehicle used in both the traditional LLNA and the LLNA: DA tests for 2-mercaptobenzothiazole. The positive result for 2-mercaptobenzothiazole reported in the ICCVAM-recommended LLNA performance standards was based on one LLNA experiment that tested the substance at 1%, 3%, and 10% (Gerberick et al. 2005). By comparison, the negative result for 2-mercaptobenzothiazole obtained with the LLNA: DA test method was based on one LLNA: DA experiment that tested the substance at 10%, 25%, and 50% (Idehara et al. 2008). The highest dose tested for 2-mercaptobenzothiazole in the traditional LLNA was the lowest dose tested in the LLNA: DA (10%) and resulted in an SI of 8.6 versus 2.0, respectively.

Notably, a review of the original LLNA: DA laboratory records for 2-mercaptobenzothiazole indicated that the concurrent positive control (10% eugenol in DMF) failed to yield an SI ≥ 3.0. Consequently the test method developers should have repeated the test for 2-mercaptobenzothiazole to ensure that the result obtained was correctly classified as negative and not the result of a failed experiment. This could explain the discordant result obtained between the traditional LLNA and the LLNA: DA test method for this test substance.

The second discordant substance, methyl methacrylate, was classified as a sensitizer based on traditional LLNA results (EC3 = 90%) but as a nonsensitizer based on LLNA: DA data. As indicated in **Table C-4**, acetone: olive oil (AOO; 4:1) was the vehicle used in both the traditional LLNA and the LLNA: DA tests for methyl methacrylate. The positive result for methyl methacrylate reported in the ICCVAM-recommended LLNA performance standards was based on one LLNA experiment that tested the substance at 10%, 30%, 50%, and 100% (Betts et al. 2006). By comparison, the negative result for methyl methacrylate obtained with the LLNA: DA test method was based on one LLNA: DA experiment that tested the substance at 25%, 50%, 75%, and 100% (Idehara unpublished). The highest dose tested for methyl methacrylate in the traditional LLNA was the same in the LLNA: DA (100%) and resulted in an SI of 3.6 versus 1.8, respectively.

[11] http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm.

As shown in **Table C-4**, when compared to the traditional LLNA, the LLNA: DA at SI ≥ 3.0 predicted the same sensitization for all three of the optional reference substances tested. The optional reference substances, SLS and ethylene glycol dimethacrylate, were categorized as nonsensitizers based on GP and human data but as sensitizers by the LLNA: DA. Thus, similar to the traditional LLNA, these substances were false positive in the LLNA: DA. SLS was tested in the same vehicle (DMF) in both the traditional LLNA and the LLNA: DA. In addition, the positive results for SLS reported in the ICCVAM-recommended LLNA performance standards were based on five LLNA studies that tested SLS at 1%, 2.5%, 5%, 10%, and 20% (Loveless et al. 1996). In comparison, the positive result for SLS obtained with the LLNA: DA test method was based on one LLNA: DA experiment that tested the substance at 1%, 2.5%, 5%, and 10% (Idehara et al. 2008). The EC3 values for SLS in the traditional LLNA (8.1%) and the LLNA: DA (6.9%) were comparable. In addition, ethylene glycol dimethacrylate was tested in the same vehicle (methyl ethyl ketone) in both the traditional LLNA and the LLNA: DA. The positive result for ethylene glycol dimethacrylate reported in the ICCVAM-recommended LLNA performance standards was based on one LLNA study that tested the substance at 10%, 25%, and 50% (Gerberick et al. 2005). In comparison, the positive result for ethylene glycol dimethacrylate obtained with the LLNA: DA test method was based on one LLNA: DA experiment that also tested the substance at 10%, 25%, and 50% (Idehara unpublished). The EC3 values for ethylene glycol dimethacrylate in the traditional LLNA (28%) and the LLNA: DA (34%) were comparable.

Lastly, the optional reference substance, nickel (II) chloride, was categorized as a sensitizer based on GP and human data but as a nonsensitizer by the LLNA: DA. Thus, similar to the traditional LLNA, this substance was false negative in the LLNA: DA. Nickel (II) chloride was tested in the same vehicle (dimethyl sulfoxide [DMSO]) in both the traditional LLNA and the LLNA: DA. In addition, the negative results for nickel (II) chloride reported in the ICCVAM-recommended LLNA performance standards were based on two independent LLNA studies that tested the substance at 0.5%, 1%, and 2.5% (Basketter et al. 1999a) and at 1%, 2.5%, and 5% (Basketter and Scholes 1992). In comparison, the negative result for nickel (II) chloride obtained with the LLNA: DA test method was based on one LLNA: DA experiment that tested the substance at 2.5%, 5%, and 10% (Idehara unpublished). The highest dose tested for nickel (II) chloride in the traditional LLNA was the same in the LLNA: DA (5%) and resulted in an SI of 2.4 versus 1.3, respectively.

Table C-4 Performance of the LLNA: DA (SI ≥ 3.0) Compared to the ICCVAM-recommended LLNA Performance Standards Reference Substances[1] (Sorted by Traditional LLNA EC3 Value)

Substance Name	ICCVAM-recommended LLNA Performance Standards				LLNA: DA[2]			
	Vehicle	Result	EC3 (%) (Max. SI)[3]	N[4]	Vehicle	Result	EC3 (%) (Max. SI)[3]	N[4]
5-Chloro-2-methyl-4-isothiazolin-3-one	DMF	+	0.009 (27.7)	1	DMF	+	0.03 (7.5)	1
2,4-Dinitrochlorobenzene	AOO	+	0.049 (43.9)	15	AOO	+	0.08 (15.1)	11
4-Phenylenediamine	AOO	+	0.110 (26.4)	6	AOO	+	0.07 (5.1)	1
Cobalt chloride	DMSO	+	0.600 (7.2)	2	DMSO	+	1.27 (20.6)	5

continued

Table C-4 Performance of the LLNA: DA (SI ≥ 3.0) Compared to the ICCVAM-recommended LLNA Performance Standards Reference Substances[1] (Sorted by Traditional LLNA EC3 Value) (continued)

Substance Name	ICCVAM-recommended LLNA Performance Standards				LLNA: DA[2]			
	Vehicle	Result	EC3 (%) (Max. SI)[3]	N[4]	Vehicle	Result	EC3 (%) (Max. SI)[3]	N[4]
Isoeugenol	AOO	+	1.540 (31.0)	47	AOO	+	2.94 (12.4)	4
2-Mercaptobenzothiazole	***DMF***	**+**	***1.700 (8.6)***	***1***	***DMF***	**-**	***NA (2.0)***	***1***
Citral	AOO	+	9.170 (20.5)	6	AOO	+	15.63 (4.4)	1
Hexyl cinnamic aldehyde	AOO	+	9.740 (20.0)	21	AOO	+	11.10 (10.2)	18
Eugenol	AOO	+	10.090 (17.0)	11	AOO	+	4.50 (7.1)	1
Phenyl benzoate	AOO	+	13.600 (11.1)	3	AOO	+	2.26 (4.2)	1
Cinnamic alcohol	AOO	+	21.000 (5.7)	1	AOO	+	21.34 (5.7)	1
Imidazolidinyl urea	DMF	+	24.000 (5.5)	1	DMF	+	18.77 (4.7)	1
Methyl methacrylate	***AOO***	**+**	***90.000 (3.6)***	***1***	***AOO***	**-**	***NA (1.8)***	***1***
Chlorobenzene	AOO	-	NA (1.7)	1	AOO	-	NA (2.4)	1
Isopropanol	AOO	-	NA (1.7)	1	AOO	-	NA (2.0)	11
Lactic acid	DMSO	-	NA (2.2)	1	DMSO	-	NA (1.1)	5
Methyl salicylate	AOO	-	NA (2.9)	9	AOO	-	NA (1.8)	4
Salicylic acid	AOO	**-**	NA (2.5)	1	AOO	-	NA (2.0)	1
Sodium lauryl sulfate	DMF	FP	8.1 (8.9)	5	DMF	+	6.88 (3.4)	1
Ethylene glycol dimethylacrylate	MEK	FP	28.000 (7.0)	1	MEK	+	34.03 (4.5)	1
Xylene	AOO	FP	95.800 (3.1)	1	NT	NT	NT	NT
Nickel (II) chloride	DMSO	FN	NA (2.4)	2	DMSO	-	NA (1.3)	1

Bolded and italicized text highlights discordant LLNA: DA vs. traditional LLNA test results.

Abbreviations: AOO = acetone: olive oil (4:1); DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; EC3 = estimated concentration needed to produce a stimulation index of three; FN = false negative in traditional LLNA when compared to guinea pig and/or human results; FP = false positive in traditional LLNA when compared to guinea pig and/or human results; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; Max. = maximum; MEK = methyl ethyl ketone; NA = not applicable (stimulation index < 3.0); NT = not tested; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

[1] From *Recommended Performance Standards: Murine Local Lymph Node Assay* (ICCVAM 2009; available at: http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm). The table lists the 18 required reference substances first (sorted from lowest to highest EC3 value), followed by the four optional reference substances (sorted from lowest to highest EC3 value).

[2] Substances tested in LLNA: DA intralaboratory validation study (Idehara et al. 2008; Idehara unpublished) and/or two-phased interlaboratory validation study (Omori et al. 2008).

[3] Based on mean EC3 value when more than one value was available. Numbers in parentheses indicate the maximum SI.

[4] Number of LLNA studies from which data were obtained.

Table C-5 provides the range and characteristics for 44 substances tested in the LLNA: DA based on sufficient traditional LLNA data. These substances are compared to the range of 18 required reference substances included on the ICCVAM-recommended LLNA performance standards reference substances list (ICCVAM 2009). The table indicates that the range of the substances tested in the LLNA: DA is similar to that included in the performance standards list. In general, there is a proportionally increased number of substances tested in the LLNA: DA in each of the categories included in the table.

Table C-5 Characteristics of the Substances Tested in the LLNA: DA Compared to the ICCVAM-recommended LLNA Performance Standards Reference Substances[1]

EC3 Range in the Traditional LLNA (%)	No. Substances	Solid/ Liquid	Actual EC3 Range (%)[2]	Human Data	Peptide Reactivity (High/Mod/Min/Low/Unk)[3]
<0.1	**5**	**3/3[4]**	**0.009-0.083**	**5**	**4/0/0/0/1**
	2	1/1	0.009-0.049	2	2/0/0/0/0
≥0.1 to <1	**6**	**5/1**	**0.110-0.600**	**6**	**1/2/0/0/3**
	2	2/0	0.110-0.600	2	0/0/0/0/2
≥1 to <10	**11**	**6/5**	**1.540-9.740**	**10**	**4/0/3/1/3**
	4	1/3	1.540-9.740	4	2/0/1/0/1
≥10 to <100	**10**	**4/6**	**10.090-90.000**	**10**	**2/1/0/1/6**
	5	3/2	10.090-90.000	5	0/1/0/0/4
Negative	**12**	**7/5**	**NA**	**10**	**0/0/8/1/3**
	5	1/4	NA	3	0/0/2/0/3
Overall	**44**	**25/20[4]**	**0.009-90.000**	**41**	**11/3/11/3/16**
	18	8/10	0.009-90.000	16	4/1/3/0/10

Boldface represents characteristics of the LLNA: DA database, which includes the 44 substances with adequate traditional LLNA data, tested in the intralaboratory validation study (Idehara et al. 2008; Idehara unpublished) and/or the two-phased interlaboratory validation study (Omori et al. 2008).

Abbreviations: EC3 = estimated concentration needed to produce a stimulation index of three; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP Content; NA = not applicable because maximum stimulation index < 3.0; No. = number; Min = minimal; Mod = moderate; Unk = unknown.

[1] From *Recommended Performance Standards: Murine Local Lymph Node Assay* (ICCVAM 2009; available at: http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm), based on the 18 required reference substances.

[2] Based on traditional LLNA studies for substances tested in the LLNA: DA (bold values) and for the 18 required reference substances in the ICCVAM-recommended LLNA performance standards (ICCVAM 2009).

[3] Data obtained from Gerberick et al. (2007).

[4] One substance tested in the LLNA: DA, benzalkonium chloride, is categorized as both a solid and a liquid.

6.4 Discordant Results for Accuracy Analysis Using the SI ≥ 3.0 Decision Criterion

6.4.1 Discordance Between the LLNA: DA and the Traditional LLNA

When the outcomes for the 44 substances tested in the LLNA: DA (using SI ≥ 3.0) and the traditional LLNA were compared, the classifications for four substances were different. The LLNA: DA classified 3-aminophenol, 2-mercaptobenzothiazole, methyl methacrylate, and nickel (II) sulfate hexahydrate as nonsensitizers while the traditional LLNA classified them as sensitizers (**Tables C-6** and **C-7**). These substances were tested in the same vehicle in both the LLNA: DA and the traditional LLNA tests. One commonality noted between three of the four discordant substances is that they are solids. Furthermore, the molecular weights for 3-aminophenol and methyl methacrylate are both about 100 g/mol and those for 2-mercaptobenzothiazole and nickel (II) sulfate hexahydrate are comparable at 160 g/mol (**Annex II**). In addition, all four discordant substances are considered nonirritants based on GP data (**Table C-6**).

6.4.2 Discordance Among the LLNA: DA, the Traditional LLNA, and/or the Guinea Pig Test

When analyses were restricted to the 40 substances with unequivocal LLNA: DA, traditional LLNA, and GP data, the LLNA: DA at SI ≥ 3.0 classified three substances differently compared with the traditional LLNA (**Table C-6**). 2-Mercaptobenzothiazole, methyl methacrylate, and nickel (II) sulfate hexahydrate were identified as nonsensitizers by the LLNA: DA while the traditional LLNA and GP tests classified these substances as sensitizers. The discordant substances were tested at the same or higher concentrations in the LLNA: DA and in the traditional LLNA yet the substances were still classified as nonsensitizers (**Table C-6**). There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Annex II** for physicochemical information), range of EC3 values (based on traditional LLNA, see **Table C-2**), and potential for skin irritation (**Annex III**) as follows:

- 2-Mercaptobenzothiazole is a heterocyclic compound, methyl methacrylate is carboxylic acid, and nickel (II) sulfate hexahydrate is a metal.
- 2-Mercaptobenzothiazole and nickel (II) sulfate hexahydrate exist as solids and methyl methacrylate exists as a liquid.
- Nickel (II) sulfate hexahydrate and methyl methacrylate are soluble in water whereas 2-mercaptobenzothizole is not.

- All three discordant substances have similar molecular weights (approximately 100 to 160 g/mol).
- 2-Mercaptobenzothaizole has high peptide reactivity, whereas the peptide reactivity for methyl methacrylate and nickel (II) sulfate hexahydrate is not known.
- All three discordant substances are classified as sensitizers by the traditional LLNA (EC3 values were 90% for methyl methacrylate, 1.7% for 2-mercaptobenzothiazole, and 4.8% for nickel [II] sulfate hexahydrate).
- All three discordant substances are nonirritants based on data from GP studies (**Table C-6**).

In addition, benzalkonium chloride, ethyl acrylate, ethylene glycol dimethacrylate, resorcinol, and SLS were positive in both the LLNA: DA and the traditional LLNA, but were negative in GP tests (**Table C-6**). In contrast, nickel (II) chloride was negative in both the LLNA: DA and the traditional LLNA but was positive in GP tests. There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Annex II** for physicochemical information), and potential for skin irritation (**Annex III**) as follows:

- Benzalkonium chloride is an amine, ethyl acrylate and ethylene glycol dimethacrylate are carboxylic acids, resorcinol is a phenol, and SLS is an alcohol, sulfur, and lipid compound; nickel (II) chloride is a metal.
- Resorcinol and SLS exist as solids in their physical state and ethyl acrylate and ethylene glycol dimethacrylate exist as liquids in their physical state, whereas benzalkonium chloride can exist in both a solid and liquid physical state; nickel (II) chloride exists as a solid in its physical state.
- These five substances have varying molecular weights (100 g/mol for ethyl acrylate, 110 g/mol for resorcinol, 171 g/mol for benzalkonium chloride, 198 g/mol for ethylene glycol dimethacrylate, and 288 g/mol for SLS); the molecular weight for nickel (II) chloride is about 130 g/mol.
- These five discordant substances are soluble in water; nickel (II) chloride is slightly soluble in water.
- Peptide reactivity is identified as minimal for resorcinol, and high for ethyl acrylate and ethylene glycol dimethacrylate, but is not identified for benzalkonium chloride and SLS; peptide reactivity for nickel (II) chloride is also not identified.
- Benzalkonium chloride and SLS have been found to be skin irritants based on results in mice, rabbits, or humans, while resorcinol is considered a nonirritant based on studies in humans, and ethyl acrylate and ethylene glycol dimethacrylate are considered nonirritants based on studies in GPs; nickel (II) chloride is identified as negative at ≤0.15% based on GP studies (**Table C-6**).

Table C-6 Discordant Results for the LLNA: DA (Using SI ≥ 3.0 for Sensitizers) Compared to Traditional LLNA and Guinea Pig Reference Data[1]

Substance Name[2]	Vehicle[3]	LLNA: DA[4]	Traditional LLNA[4]	Guinea Pig Studies[5]	Skin Irritant?
Benzalkonium chloride (0.07%)	AOO ACE[6]	+ (6.7, 2.5%)	+ (11.1, 2%)[7]	-	Irritant at 2% and 1% ACE (mice)
Ethyl acrylate (32.8%)	AOO	+ (4.2, 50%)[8]	+ (4.0, 50%)	-	Nonirritant at 0.3 Molar (GP)
Ethylene glycol dimethacrylate (28%)	MEK	+ (4.5, 50%)	+ (7.0, 50%)	-	Nonirritant at 1% (GP)

continued

Table C-6 Discordant Results for the LLNA: DA (Using SI ≥ 3.0 for Sensitizers) Compared to Traditional LLNA and Guinea Pig Reference Data[1] (continued)

Substance Name[2]	Vehicle[3]	LLNA: DA[4]	Traditional LLNA[4]	Guinea Pig Studies[5]	Skin Irritant?
Resorcinol (6.33%)	AOO	+ (4.3, 25%)[9]	+ (10.4, 50%)	-	Nonirritant at 15% (humans)
Sodium lauryl sulfate (8.08%)	DMF	+ (3.4, 10%)	+ (8.9, 20%)	-	Irritant at 20% aq. (rabbits); Irritant at 20% (humans)
Nickel (II) chloride	DMSO	- (1.3, 10%)	- (2.4, 5%)	+	Negative at ≤0.15% (GP)
2-Mercapto-benzothiazole (1.7%)	DMF	- (2.0, 50%)[9]	+ (8.6, 10%)	+	Nonirritant at 10% (GP); Nonirritant at 25% (humans)
Methyl methacrylate (90%)	AOO	- (1.8, 100%)	+ (3.6, 100%)	+	Nonirritant at 3 Molar (GP)
Nickel (II) sulfate hexahydrate (4.8%)	DMSO	- (11.8, 10%)	+ (3.1, 5%)	+	Irritant at 10% (humans); Nonirritant at 0.15% (GP)

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); aq. = aqueous; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; MEK = methyl ethyl ketone; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

[1] References for traditional LLNA, guinea pig, and skin irritant data are indicated in **Annex III-1.**

[2] Numbers in parentheses are EC3 values (estimated concentration needed to produce a stimulation index [SI] of three) for substances that are sensitizers in the traditional LLNA (see **Table C-2**).

[3] Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.

[4] Numbers in parentheses are highest SI and maximum concentration tested; highest SI is at maximum concentration test, unless otherwise noted.

[5] Based on studies using either the guinea pig maximization test or the Buehler test.

[6] Tested in AOO in LLNA: DA and ACE in traditional LLNA.

[7] Highest SI occurred at concentration 1%.

[8] Highest SI occurred at concentration 25%.

[9] Highest SI occurred at concentration 10%.

6.4.3 Discordance Among the LLNA: DA, Traditional LLNA, and/or the Human Outcome

When analyses were restricted to the 41 substances with unequivocal LLNA: DA, traditional LLNA, and human outcomes, the LLNA: DA classified four substances differently compared with the classification of the traditional LLNA (**Table C-7**). 3-Aminophenol, 2-mercaptobenzothiazole, methyl methacrylate, and nickel (II) sulfate hexahydrate were identified as nonsensitizers by the LLNA: DA while the traditional LLNA and human outcomes classified these substances as

sensitizers. All four discordant substances were tested at similar or higher concentrations in the LLNA: DA and in the traditional LLNA yet the substances were still classified as nonsensitizers (**Table C-7**). There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Annex II** for physicochemical information), range of EC3 values (based on traditional LLNA, see **Table C-2**), and potential for skin irritation (**Annex III**):

- 3-Aminophenol is an amine and phenol compound, 2-mercaptobenzothiazole is a heterocyclic compound, methyl methacrylate is a carboxylic acid, and nickel (II) sulfate hexahydrate is a metal.
- All four discordant substances exist as solids in their physical state except methyl methacrylate, which is a liquid.
- All four discordant substances are soluble in water except 2-mercaptobenzothizole.
- Molecular weights range from 100 to 167 g/mol.
- 2-Mercaptobenzothaizole has high peptide reactivity and 3-aminophenol has minimal peptide reactivity; peptide reactivity information for methyl methacrylate and nickel (II) sulfate hexahydrate is not available.
- All four discordant substances are classified as sensitizers by the traditional LLNA (EC3 values are 1.7% for 2-mercaptobenzothiazole, 3.2% for 3-aminophenol, 4.8% for nickel [II] sulfate hexahydrate, and 90% for methyl methacrylate).
- All four discordant substances are classified as nonirritants based on data from GP studies, although human data indicate that nickel (II) sulfate hexahydrate is an irritant at 10% (**Table C-7**).

In addition, the LLNA: DA predicted the same outcome for SLS as the traditional LLNA (i.e., sensitizer), but was discordant when compared to the negative human test result (**Table C-7**). Diethyl phthalate, isopropanol, nickel (II) chloride, propylparaben and sulfanilamide were also predicted similarly by the LLNA: DA and the traditional LLNA (i.e., nonsensitizers) but were discordant when compared to the positive human test result (**Table C-7**). There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Annex II** for physicochemical information), range of EC3 values (based on traditional LLNA, see **Table C-2**), and potential for skin irritation (**Annex III**):

- SLS is an alcohol, sulfur, and lipid compound; diethyl phthalate is a carboxylic acid, isopropanol is an alcohol, nickel (II) chloride is a metal, propylparaben is a phenol compound, and sulfanilamide is a cyclic hydrocarbon and sulfur compound.
- SLS exists as a solid in its physical state; diethyl phthalate and isopropanol are liquids in their physical state, whereas nickel (II) chloride, propylparaben, and sulfanilamide exist as solids in their physical state.
- These substances have varying molecular weights that range from 60 to 222 g/mol for diethyl phthalate, isopropanol, nickel (II) chloride, propylparaben, and sulfanilamide to 288 g/mol for SLS.
- SLS, diethyl phthalate, isopropanol, nickel (II) chloride, and sulfanilamide are soluble in water and propylparaben is not.
- Diethyl phthalate, isopropanol, propylparaben, and sulfanilamide have minimal peptide reactivity; peptide reactivity data for nickel (II) chloride and SLS are not available.
- SLS has been found to be a skin irritant based on results in mice, rabbits, or humans; diethyl phthalate, isopropanol, nickel (II) chloride, propylparaben, and sulfanilamide are considered negative or nonirritants based on studies in rabbits or GP (**Table C-7**).

Table C-7 Discordant Results for the LLNA: DA (Using SI ≥ 3.0 for Sensitizers) Compared to Traditional LLNA and Human Reference Data[1]

Substance Name[2]	Vehicle[3]	LLNA: DA[4]	Traditional LLNA[4]	Human Outcomes[5]	Skin Irritant?
Sodium lauryl sulfate (8.08%)	DMF	+ (3.4, 10%)	+ (8.9, 20%)	- (0/22 at 10%)	Irritant at 20% aq. (rabbits); Irritant at 20% (humans)
Diethyl phthalate	AOO	- (1.09, 100%)[6]	- (1.5, 100%)	+ (HPTA)	Negative at 100% (rabbits)
Isopropanol	AOO	- (1.97, 50%)	- (1.7, 50%)[6]	+ (case study at 0.001%)	Negative at 100% (rabbits)
Nickel (II) chloride	DMSO	- (1.3, 10%)	- (2.4, 5%)	+ (HMT, data expressed as nickel)	Negative at ≤0.15% (GP)
Propylparaben	AOO	- (1.3, 25%)	- (1.4, 25%)[7]	+ (HMT)	Nonirritant at 10% (GP)
Sulfanilamide	DMF	- (0.9, 50%)[6]	- (1.0, 50%)[8]	+ (20/25 at 25%)	Nonirritant at 25% (humans)
3-Aminophenol (3.2%)	AOO	- (2.8, 10%)	+ (5.7, 10%)	+	Nonirritant at 5% (GP)
2-Mercapto-benzothiazole (1.7%)	DMF	- (2.0, 50%)[9]	+ (8.6, 10%)	+ (24/63 at 25%)	Nonirritant at 10% (GP); Nonirritant at 25% (humans)
Methyl methacrylate (90%)	AOO	- (1.8, 100%)	+ (3.6, 100%)	+	Nonirritant at 3 M (GP)
Nickel (II) sulfate hexahydrate (4.8%)	DMSO	- (11.8, 10%)	+ (3.1, 5%)	+ (23/88 at 1%)	Irritant at 10% (humans); Nonirritant at 0.15% (GP)

Abbreviations: AOO = acetone: olive oil (4:1); aq. = aqueous; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pig; HMT = human maximization test; HPTA = human patch test allergen; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

[1] References for traditional LLNA, human, and skin irritant data are indicated in **Annex III-1.**

[2] Numbers in parentheses are EC3 values (estimated concentration needed to produce a stimulation index [SI] of three) for substances that are sensitizers in the traditional LLNA (see **Table C-2**).

[3] Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.

[4] Numbers in parentheses are highest SI and maximum concentration tested; highest SI is at maximum concentration tested, unless otherwise noted.

[5] Based on studies using either the human maximization test, inclusion of the test substance in a human patch test allergen kit, and/or published clinical case studies/reports.

[6] Highest SI occurred at concentration 25%.

[7] Highest SI occurred at concentration 5%.

[8] Highest SI occurred at concentration 10% and 25%.

[9] Highest SI occurred at concentration 10%.

6.5 Accuracy Analysis Using Single Alternative Decision Criteria

In addition to the accuracy analysis using SI ≥ 3.0 to classify substances as sensitizers, other decision criteria were evaluated on the LLNA: DA test method performance, using the traditional LLNA (SI ≥ 3.0) as the comparative test (**Annex III**). The performance characteristics presented in this section are for 14 decision criteria that were used to determine whether the skin sensitization potential for the substances were positive (i.e., sensitizing) or negative (i.e., nonsensitizing). The substances evaluated were the 44 substances discussed in **Section 6.1** with both LLNA: DA and adequate comparative traditional LLNA data. The decision criteria analyzed included the following:

1. SI values ≥1.3, ≥1.5, ≥1.8, ≥2.0, ≥2.5, ≥3.0, ≥3.5, ≥4.0, ≥4.5, or ≥5.0
2. Log-transformed ATP values of treated groups statistically different from control group based on analysis of variance (ANOVA) with a post-hoc Dunnett's test, when multiple treatment groups were tested, or Student's *t*-test when there was only one dosed group
3. Mean ATP values of treated groups ≥95% confidence interval (CI) of the control group mean
4. Mean ATP values of treated groups ≥2 standard deviations (SD) or ≥3 SD from the control group mean

Multiple tests were available for 14 substances tested with the LLNA: DA. The results for each of these substances were combined so that each substance was represented by one positive or negative result for each criterion evaluated for the accuracy analyses. The results were combined in three ways and a separate accuracy analysis was performed for each approach.

1. The positive/negative outcome for each substance was the most prevalent outcome for each criterion. If the number of positive and negative outcomes were equal, the most conservative (i.e., positive) result was used for the accuracy analyses.
2. The positive/negative outcome for each substance for each criterion was determined by the outcome of the test with the highest maximum SI of the multiple tests.
3. The positive/negative outcome for each substance was determined by the outcome of the test with the lowest maximum SI of the multiple tests.

The analysis using the most prevalent outcome for substances with multiple tests is presented in this section; the analyses using the highest maximum SI and the lowest maximum SI are included in **Annex V**.

When combining multiple test results for a single substance based on the most prevalent outcome, using the decision criterion of SI ≥ 3.0 to identify sensitizers, the 44 substances analyzed yielded an accuracy of 91% (40/44), a sensitivity of 88% (28/32), a specificity of 100% (12/12), a false positive rate of 0% (0/12), and a false negative rate of 13% (4/32) (**Table C-8)**. The decision criterion of SI ≥ 2.5 was similar to SI ≥ 3.0 in its performance characteristics. In comparison, the decision criteria using higher SI values, SI ≥ 3.5 to SI ≥ 5.0, decreased performance except for specificity, which remained at 100% (12/12), and the false positive rate, which remained at 0% (0/12) (**Figure C-1** and **Table C-8**). Specifically, at SI ≥ 5.0, accuracy decreased to 57% (25/44) and the false negative rate increased to 59% (19/32).

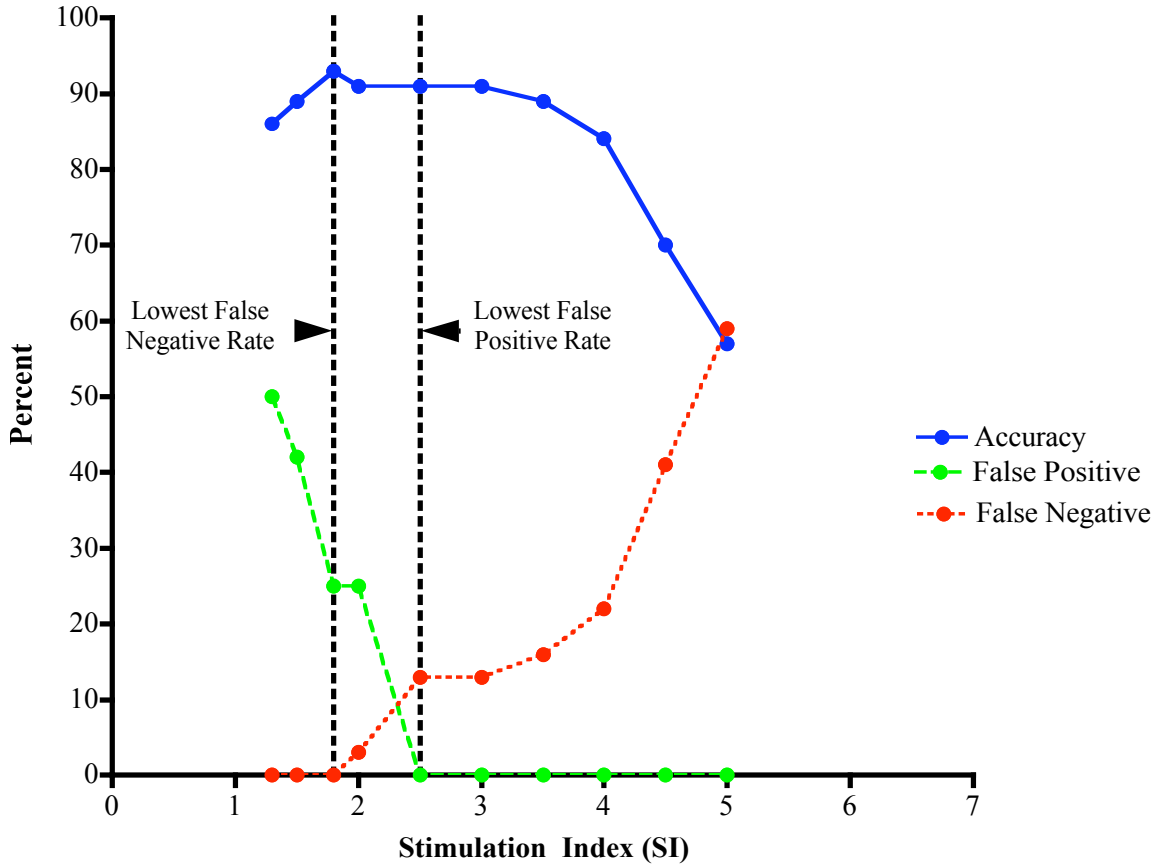
The decision criteria using lower SI values, SI ≥ 1.5 and SI ≥ 1.3, also decreased performance compared to SI ≥ 3.0 except for sensitivity, which increased to 100% (32/32), and the false negative rate, which decreased to 0% (0/32) (**Figure C-1** and **Table C-8**). Further, compared to SI ≥ 3.0, the lower SI cutoff of 2.0 had the same accuracy (91% [40/44]) but had an increased sensitivity of 97% (31/32), although specificity decreased to 75% (9/12) and the false positive rate increased to 25% (3/12) while the false negative rate decreased to 3% (1/32) (**Figure C-1** and **Table C-8**). Notably, the SI decision criterion that exhibited optimum performance characteristics compared to SI ≥ 3.0 was SI ≥ 1.8 (**Figure C-1** and **Table C-8**). Compared to SI ≥ 3.0, the lower SI cutoff of 1.8 had increased accuracy (93% [41/44]) and sensitivity (100% [32/32]), although specificity decreased to 75% (9/12) and the false positive rate increased to 25% (3/12) while the false negative rate decreased to 0% (0/32) (**Figure C-1** and **Table C-8**).

Use of ANOVA and summary statistics (i.e., mean ATP values of treated groups ≥95% confidence interval of the control group mean, or ≥2 or 3 SD from the control group mean), yielded accuracy values of 75 to 84%, with sensitivity values of 88 to 100%, and false negative rates of 0 to 13%. The specificity for these criteria ranged from 8 to 58% and the false positive rates were 42 to 92%. None of the statistical criterion evaluated exhibited increased performance characteristics when compared to SI ≥ 3.0 (**Table C-8**).

An evaluation to determine the robustness of the optimum SI ≥ 1.8 criterion indicated that the SI was quite stable. Taking different samples of the data as training and validation sets had relatively little impact on the cutoff SI criterion or on the resulting number of false or false negative results (see **Annex VI**). Since the decision criterion of SI ≥ 1.8 showed optimum performance (i.e., increased accuracy and sensitivity, and decreased false negative rate compared to SI ≥ 3.0), it was further compared to SI ≥ 3.0 for accuracy against GP and human data (**Table C-9)**. When the LLNA: DA was compared to GP outcomes for substances with LLNA: DA, traditional LLNA, and GP data (40 substances), SI ≥ 1.8 had increased accuracy (80% [32/40] vs. 78% [31/40]), increased sensitivity (96% [25/26] vs. 85% [22/26]) and decreased specificity (50% [7/14] vs. 64% [9/14]) when compared with SI ≥ 3.0. Accordingly, the false positive rate was increased (50% [7/14] vs. 36% [5/14]) and the false negative rate was decreased (4% [1/26] vs. 15% [4/26]) for SI ≥ 1.8 compared to SI ≥ 3.0. The overall performance of the LLNA: DA (SI ≥ 1.8 or SI ≥ 3.0) compared to the traditional LLNA (SI ≥ 3.0) to predict GP outcomes was less (see **Table C-9**).

When the LLNA: DA was compared to human outcomes for substances with LLNA: DA, traditional LLNA, and human data (41 substances), SI ≥ 1.8 increased the accuracy (80% [33/41] vs. 76% [31/41]) and sensitivity (86% [30/35] vs. 74% [26/35]) and decreased the specificity (50% [3/6] vs. 83% [5/6]) when compared with SI ≥ 3.0. Accordingly, the false positive rate was increased (50% [3/6] vs. 17% [1/6]) and the false negative rate was decreased (14% [5/35] vs. 26% [9/35]). The overall performance of the LLNA: DA (SI ≥ 1.8 or SI ≥ 3.0) compared to the traditional LLNA (SI ≥ 3.0) to predict human outcomes was less (see **Table C-9**).

Figure C-1 Performance of the LLNA: DA for 44 Substances Compared to the Traditional LLNA in Predicting Skin Sensitization Potential Using Alternative SI Based on the Most Prevalent Outcome for Substances with Multiple Tests



As compared to traditional LLNA results, the lines show the change in performance characteristics for the LLNA: DA with the SI cutoff used to identify sensitizers. This analysis used LLNA: DA and traditional LLNA results for 44 substances (32 traditional LLNA sensitizers and 12 traditional LLNA nonsensitizers). For the 14 substances with multiple test results in the LLNA: DA, the results for each substance were combined by using the most prevalent outcome. The solid line shows accuracy, the dashed line shows the false positive rate, and the dotted line shows the false negative rate.

Abbreviations: LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

Table C-8 Performance of the LLNA: DA for 44 Substances Compared to the Traditional LLNA in Predicting Skin Sensitization Potential Using Alternative Decision Criteria Based on the Most Prevalent Outcome for Substances with Multiple Tests

Alternate Criterion	N[1]	Accuracy % (No.[2])	Sensitivity % (No.[2])	Specificity % (No.[2])	False Positive Rate % (No.[2])	False Negative Rate % (No.[2])	Positive Predictivity % (No.[2])	Negative Predictivity % (No.[2])
Statistics[3]	44	84 (37/44)	94 (30/32)	58 (7/12)	42 (5/12)	6 (2/32)	86 (30/35)	78 (7/9)
≥95% CI[4]	44	75 (33/44)	100 (32/32)	8 (1/12)	92 (11/12)	0 (0/32)	74 (32/43)	100 (1/1)
≥2 SD[5]	44	77 (34/44)	91 (29/32)	42 (5/12)	58 (7/12)	9 (3/32)	81 (29/36)	63 (5/8)
≥3 SD[6]	44	80 (35/44)	88 (28/32)	58 (7/12)	42 (5/12)	13 (4/32)	85 (28/33)	64 (7/11)
SI ≥ 5.0	44	57 (25/44)	41 (13/32)	100 (12/12)	0 (0/12)	59 (19/32)	100 (13/13)	39 (12/31)
SI ≥ 4.5	44	70 (31/44)	59 (19/32)	100 (12/12)	0 (0/12)	41 (13/32)	100 (19/19)	48 (12/25)
SI ≥ 4.0	44	84 (37/44)	78 (25/32)	100 (12/12)	0 (0/12)	22 (7/32)	100 (25/25)	63 (12/19)
SI ≥ 3.5	44	89 (39/44)	84 (27/32)	100 (12/12)	0 (0/12)	16 (5/32)	100 (27/27)	71 (12/17)
SI ≥ 3.0	*44*	*91 (40/44)*	*88 (28/32)*	*100 (12/12)*	*0 (0/12)*	*13 (4/32)*	*100 (28/28)*	*75 (12/16)*
SI ≥ 2.5	44	91 (40/44)	88 (28/32)	100 (12/12)	0 (0/12)	13 (4/32)	100 (28/28)	75 (12/16)
SI ≥ 2.0	44	91 (40/44)	97 (31/32)	75 (9/12)	25 (3/12)	3 (1/32)	91 (31/34)	90 (9/10)
SI ≥ 1.8	**44**	**93 (41/44)**	**100 (32/32)**	**75 (9/12)**	**25 (3/12)**	**0 (0/32)**	**91 (32/35)**	**100 (9/9)**
SI ≥ 1.5	44	89 (39/44)	100 (32/32)	58 (7/12)	42 (5/12)	0 (0/32)	86 (32/37)	100 (7/7)
SI ≥ 1.3	44	86 (38/44)	100 (32/32)	50 (6/12)	50 (6/12)	0 (0/32)	84 (32/38)	100 (6/6)

Italicized text indicates the decision criterion chosen by the LLNA: DA validation study team; Bold text indicates the single decision criterion that had an overall increased performance in predicting skin sensitization potential when compared to the traditional LLNA.

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP Content; No. = number; SD = standard deviation; SI = stimulation index.

[1] N = Number of substances included in this analysis.

[2] The proportion on which the percentage calculation is based.

[3] Analysis of variance for difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The ATP data were log-transformed prior to statistical analysis. For analysis of variance, significance at $p < 0.05$ was further tested by Dunnett's test.

[4] The mean ATP of at least one treatment group was outside the 95% confidence interval for the mean ATP of the vehicle control group.

[5] The mean ATP of at least one treatment group was greater than 2 SD from the mean ATP of the vehicle control group.

[6] The mean ATP of at least one treatment group was greater than 3 SD from the mean ATP of the vehicle control group.

Table C-9 Performance of the LLNA: DA in Predicting Skin Sensitization Potential Comparing Decision Criteria of SI ≥ 3.0 versus SI ≥ 1.8 Based on the Most Prevalent Outcome for Substances with Multiple Tests

Comparison	n[1]	Decision Criterion	Accuracy % (No.[2])	Sensitivity % (No.[2])	Specificity % (No.[2])	False Positive Rate % (No.[2])	False Negative Rate % (No.[2])	Positive Predictivity % (No.[2])	Negative Predictivity % (No.[2])
LLNA: DA vs. Traditional LLNA	44	SI ≥ 3.0	91 (40/44)	88 (28/32)	100 (12/12)	0 (0/12)	13 (4/32)	100 (28/28)	75 (12/16)
		SI ≥ 1.8	93 (41/44)	100 (32/32)	75 (9/12)	25 (3/12)	0 (0/32)	91 (32/35)	100 (9/9)
Substances with LLNA: DA, Traditional LLNA, and GP Data									
LLNA: DA vs. Traditional LLNA	40	SI ≥ 3.0	93 (37/40)	90 (27/30)	100 (10/10)	0 (0/10)	10 (3/30)	100 (27/27)	77 (10/13)
		SI ≥ 1.8	95 (38/40)	100 (30/30)	80 (8/10)	20 (2/10)	0 (0/30)	94 (30/32)	100 (8/8)
LLNA: DA vs. GP[3]	40	SI ≥ 3.0	78 (31/40)	85 (22/26)	64 (9/14)	36 (5/14)	15 (4/26)	81 (22/27)	69 (9/13)
		SI ≥ 1.8	80 (32/40)	96 (25/26)	50 (7/14)	50 (7/14)	4 (1/26)	78 (25/32)	88 (7/8)
Traditional LLNA vs. GP[3]	40	SI ≥ 3.0	85 (34/40)	96 (25/26)	64 (9/14)	36 (5/14)	4 (1/26)	83 (25/30)	90 (9/10)
Substances with LLNA: DA, Traditional LLNA, and Human Data									
LLNA: DA vs. Traditional LLNA	41	SI ≥ 3.0	90 (37/41)	87 (27/31)	100 (10/10)	0 (0/10)	13 (4/31)	100 (27/27)	71 (10/14)
		SI ≥ 1.8	95 (39/41)	100 (31/31)	80 (8/10)	20 (2/10)	0 (0/31)	94 (31/33)	100 (8/8)
LLNA: DA vs. Human[4]	41	SI ≥ 3.0	76 (31/41)	74 (26/35)	83 (5/6)	17 (1/6)	26 (9/35)	96 (26/27)	36 (5/14)
		SI ≥ 1.8	80 (33/41)	86 (30/35)	50 (3/6)	50 (3/6)	14 (5/35)	91 (30/33)	38 (3/8)
Traditional LLNA vs. Human[4]	41	SI ≥ 3.0	85 (35/41)	86 (30/35)	83 (5/6)	17 (1/6)	14 (5/35)	97 (30/31)	50 (5/10)

Abbreviations: GP = guinea pig skin sensitization outcomes; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; No. = number; SI = stimulation index; vs. = versus.

[1] n = Number of substances included in this analysis.

[2] The proportion on which the percentage calculation is based.

[3] GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.

[4] Human refers to outcomes obtained by studies conducted using the human maximization test, inclusion of the test substance in a human patch test allergen kit, and/or published clinical case studies/reports.

6.6 Discordant Results for Accuracy Analysis Using Single Alternative Decision Criteria

This section discusses the discordant results obtained for the analyses using the alternative decision criteria shown in **Tables C-8** and **C-9,** in order to provide a comparison to the discordant substances identified when using the decision criterion of SI ≥ 3.0 to identify sensitizers. Discordant results for the alternative decision criteria are first discussed in general using the traditional LLNA as the reference test (**Section 6.6.1**) and then discordant results for SI ≥ 1.8, the single optimized alternative decision criterion, are discussed using the traditional LLNA, GP, and human outcomes as references (**Section 6.6.2**).

6.6.1 Discordant Results Using Single Alternative Decision Criteria Compared with the Traditional LLNA

Table C-10 shows how the number and identity of discordant substances changes with the alternative decision criteria when using the most prevalent outcome for the substances with multiple tests. Using SI ≥ 2.0 as the decision criterion resulted in three nonsensitizers in the traditional LLNA (chlorobenzene, hexane, and salicylic acid) being misclassified as sensitizers in the LLNA: DA. Also, methyl methacrylate, a sensitizer in the traditional LLNA, was misclassified as a nonsensitizer in the LLNA: DA. Using SI ≥ 1.8 as the decision criterion still resulted in chlorobenzene, hexane, and salicylic acid being misclassified as sensitizers in the LLNA: DA compared to the traditional LLNA, although methyl methacrylate was no longer misclassified as a nonsensitizer in the LLNA: DA compared to SI ≥ 2.0. As the SI decision criterion was further reduced to SI ≥ 1.5 and SI ≥ 1.3, two additional substances, 1-bromobutane and methyl salicylate, were also misclassified as sensitizers when compared to traditional LLNA results. In addition, using SI ≥ 1.3 also misclassified nickel (II) chloride as a sensitizer in the LLNA: DA compared to the traditional LLNA. Increasing the SI cutoff to values greater than three increased the number of sensitizers that were misclassified as nonsensitizers. At SI ≥ 5.0, 19 substances were discordant. As **Table C-10** shows, all 19 substances were sensitizers in the LLNA but misclassified as nonsensitizers in the LLNA: DA.

Use of a statistical test (i.e., ANOVA or *t*-test) to identify sensitizers misclassified two sensitizers in the traditional LLNA (2-mercaptobenzothiazole and methyl methacrylate) as nonsensitizers in the LLNA: DA and five nonsensitizers (1-bromobutane, chlorobenzene, hexane, salicylic acid, and sulfanilamide) as sensitizers. Use of summary statistics (i.e., ≥95% CI, ≥2 SD or ≥3 SD) generally misclassified nonsensitizers in the traditional LLNA as sensitizers in the LLNA: DA. Specifically, using ≥3 SD of vehicle control mean misclassified five nonsensitizers as sensitizers: 1-bromobutane, chlorobenzene, hexane, nickel (II) chloride, and propylparaben. Using treatment group absorbance ≥2 SD of vehicle control mean misclassified the same five substances as sensitizers, as well as methyl salicylate and salicylic acid. Using the treatment group absorbance ≥95% CI of vehicle control mean misclassified all the nonsensitizers misclassified as sensitizers in the LLNA: DA when using either ≥3 SD or ≥2 SD of vehicle control mean, as well as four additional substances: diethyl phthalate, dimethyl isophthalate, isopropanol, and lactic acid. In some instances, use of summary statistics (i.e., ≥95% CI, ≥2 SD or ≥3 SD) misclassified sensitizers in the traditional LLNA as nonsensitizers in the LLNA: DA. Using ≥3 SD of vehicle control mean misclassified four traditional LLNA sensitizers as LLNA: DA nonsensitizers: butyl glycidyl ether, ethyl acrylate, methyl methacrylate, and propyl gallate. Using treatment group absorbance ≥2 SD of vehicle control mean only misclassified ethyl acrylate and propyl gallate as nonsensitizers in the LLNA: DA compared to the traditional LLNA and using the treatment group absorbance ≥95% CI did not misclassify any traditional LLNA sensitizers as LLNA: DA nonsensitizers.

6.6.2 Discordant Results for Accuracy Analysis Using a Single Optimized Alternative Decision Criterion (SI ≥ 1.8)

When analyses were restricted to the 40 substances with unequivocal LLNA: DA, traditional LLNA, and GP data based on an SI ≥ 1.8, the LLNA: DA classified two substances (chlorobenzene and salicylic acid) differently compared with the classification of the traditional LLNA (**Table C-11**). Chlorobenzene and salicylic acid were classified as sensitizers in the LLNA: DA and as nonsensitizers by both the traditional LLNA and GP outcomes. In contrast, benzalkonium chloride, ethyl acrylate, ethylene glycol dimethacrylate, resorcinol, and sodium lauryl sulfate were identified as sensitizers by the LLNA: DA similar to the traditional LLNA but as nonsensitizers based on GP outcomes. Further, nickel (II) chloride was identified as a nonsensitizer by the LLNA: DA similar to the traditional LLNA but as a sensitizer based on GP outcomes. There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Annex II** for physicochemical information), range of EC3 values (based on traditional LLNA, see **Table C-2**), and potential for skin irritation (**Annex III**) as follows:

- Chlorobenzene is a halogenated hydrocarbon compound and salicylic acid is a phenol and carboxylic acid; benzalkonium chloride is an amine (also an onium compound), ethyl acrylate and ethylene glycol dimethacrylate are carboxylic acids, resorcinol is a phenol, and SLS is an alcohol, sulfur, and lipid compound; nickel (II) chloride is a metal.
- Chlorobenzene exists as a liquid and salicylic acid exists as a solid in its physical state; benzalkonium chloride can exist in both a solid and liquid physical state, whereas ethyl acrylate and ethylene glycol dimethacrylate are liquids, and resorcinol and SLS are solids; nickel (II) chloride is a solid.
- Chlorobenzene has a molecular weight of 113 g/mol and salicylic acid has a molecular weight of 138 g/mol; the five substances that are concordant with the traditional LLNA but discordant with GP outcomes have varying molecular weights that range from 100 g/mol for ethyl acrylate, 110 g/mol for resorcinol, 171 g/mol for benzalkonium chloride, and 198 g/mol for ethylene glycol dimethacrylate to 288 g/mol for SLS; the molecular weight for nickel (II) chloride is 130 g/mol.
- All the discordant substances are soluble in water.
- Chlorobenzene has minimal peptide reactivity while peptide reactivity data for salicylic acid are not available; the peptide reactivity for resorcinol is identified as minimal, and that for ethyl acrylate and ethylene glycol dimethacrylate is high while peptide reactivity data for benzalkonium chloride and SLS are not available; peptide reactivity data for nickel (II) chloride are not available.
- Benzalkonium chloride (EC3 = 0.07%), ethyl acrylate (EC3 = 32.8%), ethylene glycol dimethacrylate (EC3 = 28%), resorcinol (EC3 = 6.33%), and SLS (EC3 = 8.08%) are identified as sensitizers by the traditional LLNA.
- Chlorobenzene has low irritancy potential assumed based on clinical literature while salicylic acid is an irritant at 20% in mice; benzalkonium chloride and SLS have been found to be skin irritants based on results in mice, rabbits, or humans and ethyl acrylate, ethylene glycol dimethacrylate, and resorcinol are considered nonirritants based on studies in humans or GP; nickel (II) chloride is considered a negative at ≤0.15% based on GP data (**Table C-11**).

When analyses were restricted to the 40 substances with unequivocal LLNA: DA, traditional LLNA, and human outcomes based on an SI ≥ 1.8, the LLNA: DA classified two substances (hexane and salicylic acid) differently compared with the classification of the traditional LLNA (**Table C-12**). Hexane and salicylic acid were classified as sensitizers in the LLNA: DA and as nonsensitizers by both the traditional LLNA and human outcomes. Further, SLS was classified as a sensitizer by the LLNA: DA and traditional LLNA but as a nonsensitizer based on human outcomes. In contrast,

diethyl phthalate, isopropanol, nickel (II) chloride, propylparaben, and sulfanilamide were all classified as nonsensitizers by the LLNA: DA and the traditional LLNA but as sensitizers based on human outcomes (**Table C-12**). In instances where the substances were discordant in the LLNA: DA compared to the traditional LLNA, the discordant substances were tested at the same maximum concentration. There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Annex II** for physicochemical information), range of EC3 values (based on traditional LLNA, see **Table C-2**), and potential for skin irritation (**Annex III**):

- Hexane is an acyclic hydrocarbon compound and salicylic acid is a phenol and carboxylic acid; SLS is an alcohol, sulfur, and lipid compound; diethyl phthalate is a carboxylic acid, isopropanol is an alcohol, nickel (II) chloride is a metal, propylparaben is a phenol compound, and sulfanilamide is sulfur compound.
- Hexane is a liquid and salicylic acid is a solid; SLS is a solid; diethyl phthalate and isopropanol are liquids while nickel (II) chloride, propylparaben, and sulfanilamide are solids.
- Hexane and salicylic acid have molecular weights of 86 g/mol and 138 g/mol, respectively; the molecular weight for SLS is 288 g/mol; the other discordant substances have varying molecular weights that range from 60 g/mol for isopropanol, 130 g/mol for nickel (II) chloride, 172 g/mol for sulfanilamide, and 180 g/mol for propylparaben to 222 g/mol for diethyl phthalate.
- Hexane, salicylic acid, SLS, diethyl phthalate, isopropanol, nickel (II) chloride, and sulfanilamide are soluble in water; propylparaben is not.
- Hexane, diethyl phthalate, isopropanol, propylparaben, and sulfanilamide have minimal peptide reactivity; peptide reactivity information for salicylic acid, nickel (II) chloride, and SLS is not available.
- SLS is identified as a sensitizer by the traditional LLNA (EC3 = 8.08%).
- Hexane has been found to be an irritant at 100% in humans as has salicylic acid at 20% in mice; SLS has been found to be a skin irritant based on results in mice, rabbits, or humans; diethyl phthalate, isopropanol, nickel (II) chloride, propylparaben, and sulfanilamide are considered to be nonirritants based on studies in rabbits, GP, or humans (**Table C-12**).

Table C-10 Discordant Results for the LLNA: DA Using Alternative Decision Criteria Compared to the Traditional LLNA Based on the Most Prevalent Outcome for Substances with Multiple Tests

Discordant Substance[1]	Alternative Decision Criterion[2]													
	Statistics[3]	≥95% CI[4]	≥2 SD[5]	≥3 SD[6]	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.8	SI ≥ 1.5	SI ≥ 1.3
3-Aminophenol (3.2%)					-	-	-	-	-	-				
p-Benzoquinone (0.01%)					-	-	-							
1-Bromobutane (-)	+	+	+	+									+	+
Butyl glycidyl ether (30.9%)				-	-									
Chlorobenzene (-)	+	+	+	+							+	+	+	+
Cinnamic aldehyde (1.91%)					-									
Citral (9.17%)					-	-								
Cobalt chloride (0.6%)					-	-								
Diethyl maleate (3.6%)					-	-	-							
Diethyl phthalate (-)		+												
Dimethyl isophthalate (-)		+												
Ethyl acrylate (32.8%)			-	-	-	-								
Ethylene glycol dimethacrylate (28%)					-	-								
Formaldehyde (0.5%)					-									
Hexane (-)	+	+	+	+							+	+	+	+
Imidazolidinyl urea (24%)					-									
Isopropanol (-)		+												
Lactic acid (-)		+												

Discordant Substance[1]	Alternative Decision Criterion[2]													
	Statistics[3]	≥95% CI[4]	≥2 SD[5]	≥3 SD[6]	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.8	SI ≥ 1.5	SI ≥ 1.3
2-Mercaptobenzothiazole (1.7%)	-				-	-	-	-	-	-				
Methyl methacrylate (90%)	-		-	-	-	-	-	-	-	-	-			
Methyl salicylate (-)		+	+										+	+
Nickel (II) chloride (-)		+	+	+										+
Nickel (II) sulfate hexahydrate (4.8%)					-	-	-	-	-	-				
Phenyl benzoate (13.6%)					-	-								
Propyl gallate (0.32%)			-	-	-									
Propylparaben (-)		+	+	+										
Resorcinol (6.33%)					-	-								
Salicylic acid (-)	+	+	+								+	+	+	+
Sulfanilamide (-)	+													
Sodium lauryl sulfate (8.08%)					-	-	-	-						
Trimellitic anhydride (4.71%)					-									

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP Content; SD = standard deviation; SI = stimulation index.

[1] Compared to the traditional LLNA; traditional LLNA result in parentheses are "-" for nonsensitizers and EC3 value for sensitizers.

[2] LLNA: DA outcomes are indicated by "+" for sensitizer results and "-" for nonsensitizer results.

[3] Analysis of variance assessed differences of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The ATP data were log-transformed prior to statistical analysis. Significance by analysis of variance at $p < 0.05$ was further tested by Dunnett's test.

[4] The mean ATP of at least one treatment group was outside the 95% CI for the mean ATP of the vehicle control group.

[5] The mean ATP of at least one treatment group was greater than 2 SD from the mean ATP of the vehicle control group.

[6] The mean ATP of at least one treatment group was greater than 3 SD from the mean ATP of the vehicle control group.

Table C-11 Discordant Results for the LLNA: DA (Using SI ≥ 1.8 for Sensitizers) Compared to Traditional LLNA and GP Reference Data[1]

Substance Name[2]	Vehicle[3]	LLNA: DA[4]	Traditional LLNA[4]	Guinea Pig Studies[5]	Skin Irritant?
Chlorobenzene (-)	AOO	+ (2.4, 25%)	- (1.7, 10%)[6]	-	No data. Low irritancy potential assumed based on clinical literature.
Salicylic acid (-)	AOO	+ (2.0, 25%)	- (2.4, 25%)	-	Irritant at 20% aq. (mice)
Benzalkonium chloride (0.07%)	AOO ACE[7]	+ (6.7, 2.5%)	+ (11.1, 2%)[8]	-	Irritant at 2% and 1% ACE (mice)
Ethyl acrylate (32.8%)	AOO	+ (4.3, 50%)[6]	+ (4.0, 50%)	-	Nonirritant at 0.3 M (GP)
Ethylene glycol dimethacrylate (28%)	MEK	+ (4.5, 50%)	+ (7.0, 50%)	-	Nonirritant at 1% (GP)
Resorcinol (6.33%)	AOO	+ (4.3, 25%)[9]	+ (10.4, 50%)	-	Nonirritant at 15% (humans)
Sodium lauryl sulfate (8.08%)	DMF	+ (3.4, 10%)	+ (8.9, 20%)	-	Irritant at 20% aq. (rabbits); irritant at 20% (humans)
Nickel (II) chloride (-)	DMSO	- (1.3, 10%)	- (2.4, 5%)	+	Negative at ≤0.15% (GP)

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); aq. = aqueous ; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; MEK = methyl ethyl ketone; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

[1] References for traditional LLNA, guinea pig, and skin irritant data are indicated in **Annex III-1.**

[2] Numbers in parentheses are EC3 values (estimated concentration needed to produce a stimulation index [SI] of three) for substances that are sensitizers in the traditional LLNA (see **Table C-2**). Minus signs (-) indicate substances that were negative in the traditional LLNA.

[3] Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.

[4] Numbers in parentheses are highest SI and maximum concentration tested; highest SI is at maximum concentration tested, unless otherwise noted.

[5] Based on studies using either the guinea pig maximization test or the Buehler test.

[6] Highest SI occurred at concentration 25%.

[7] Benzalkonium chloride tested in AOO vehicle in LLNA: DA and ACE vehicle in traditional LLNA.

[8] Highest SI occurred at concentration 1%.

[9] Highest SI occurred at concentration 10%.

Table C-12 Discordant Results for the LLNA: DA (Using SI ≥ 1.8 for Sensitizers) Compared to Traditional LLNA and Human Reference Data[1]

Substance Name[2]	Vehicle[3]	LLNA: DA[4]	Traditional LLNA[4]	Human Outcomes[5]	Skin Irritant?
Hexane (-)	AOO	+ (2.3, 100%)	- (2.2, 100%)	- (0/25 at 100%)	Irritant at 100% (humans)
Salicylic acid (-)	AOO	+ (2.0, 25%)	- (2.4, 25%)	-	Irritant at 20% aq. (mice)
Sodium lauryl sulfate (8.08%)	DMF	+ (3.4, 10%)	+ (8.9, 20%)	- (0/22 at 10%)	Irritant at 20% aq. (rabbits); irritant at 20% (humans)
Diethyl phthalate (-)	AOO	- (1.09, 100%)[6]	- (1.5, 100%)	+ (HPTA)	Negative at 100% (rabbits)
Isopropanol (-)	AOO	- (1.97, 50%)	- (1.7, 50%)[7]	+ (case study at 0.001%)	Negative at 100% (rabbits)
Nickel (II) chloride (-)	DMSO	- (1.3, 10%)	- (2.4, 5%)	+	Negative at ≤0.15% (GP)
Propylparaben (-)	AOO	- (1.3, 25%)	- (1.4, 25%)[8]	+ (HMT)	Nonirritant at 10% (GP)
Sulfanilamide (-)	DMF	- (0.9, 50%)[6]	- (1.0, 50%)[9]	+	Nonirritant at 25% (humans)

Abbreviations: aq. = aqueous; AOO = acetone: olive oil (4:1); DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pig; HMT = human maximization test; HPTA = human patch test allergen; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

[1] References for traditional LLNA, human, and skin irritant data are indicated in **Annex III-1.**

[2] Numbers in parentheses are EC3 values (estimated concentration needed to produce a stimulation index [SI] of three) for substances that are sensitizers in the traditional LLNA (see **Table C-2**). Minus signs (-) indicate substances that were negative in the traditional LLNA.

[3] Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.

[4] Numbers in parentheses are highest SI and maximum concentration tested; highest SI is at maximum concentration tested, unless otherwise noted.

[5] Based on studies using either the human maximization test, inclusion of the test substance in a human patch test allergen kit, and/or published clinical case studies/reports.

[6] Highest SI occurred at concentration 25%.

[7] Highest SI occurred at concentration 10%.

[8] Highest SI occurred at concentration 5%.

[9] Highest SI occurred both at concentration 10% and at concentration 25%.

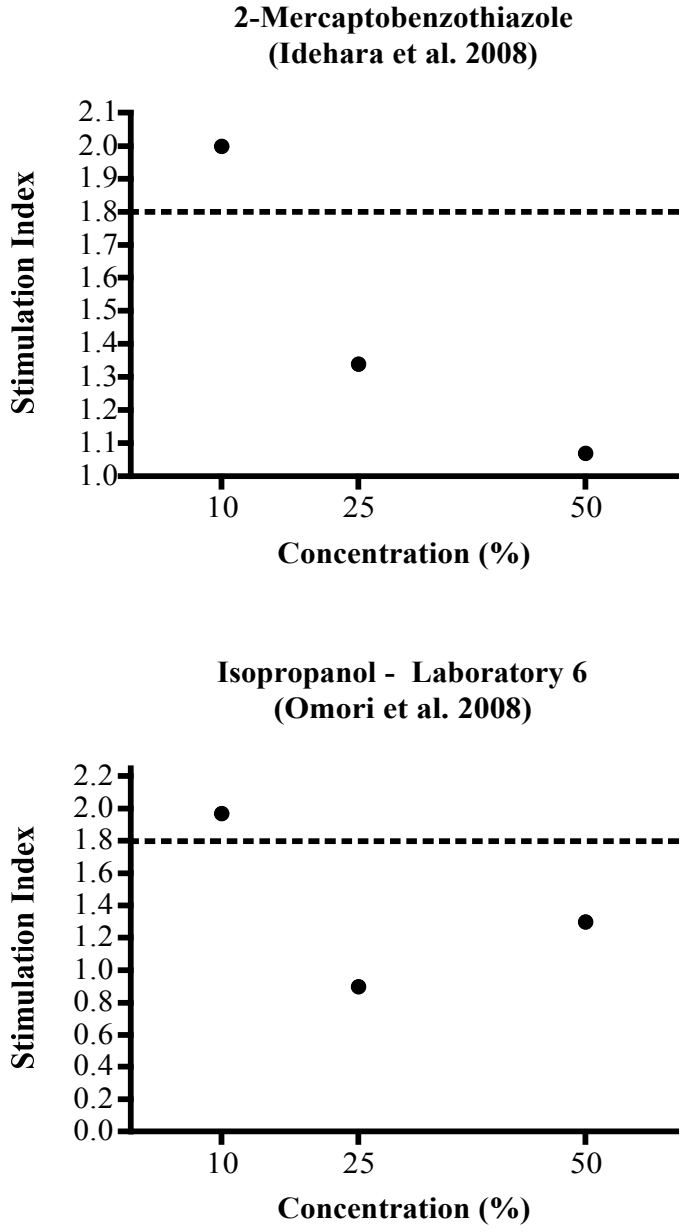
6.7 Accuracy Analysis for the Reduced LLNA: DA Using the SI ≥ 1.8 Decision Criterion

An accuracy analysis for the rLLNA: DA was performed using the optimized SI ≥ 1.8 criterion to identify sensitizers. The rLLNA: DA uses only the highest dose of the test substance that does not produce excessive skin irritation and/or systemic toxicity; the two lower dose groups are not used. The available validation database for the rLLNA: DA analysis included 123 individual tests that used multiple doses. The performance of the rLLNA: DA was evaluated by comparing the outcome of the highest dose for each test to the outcome of the same test when considering all doses tested. Using SI ≥ 1.8 to identify sensitizers, the accuracy of the rLLNA: DA was 98% (121/123), with a false positive rate of 0% (0/33) and a false negative rate of 2% (2/90). The two tests that were false negative in the rLLNA: DA were borderline positive in the multiple-dose LLNA: DA. One study that tested 2-mercaptobenzothiazole at 10%, 25%, and 50% produced a maximum SI value of 2.00 at the lowest dose tested (**Figure C-2**). The second false negative test was for isopropanol at 10%, 25%, and 50%, which produced the maximum SI of 1.97 at the lowest dose tested (**Figure C-2**).

6.8 Analyses Using Multiple Alternative Decision Criteria

As detailed in **Section 6.5,** the accuracy of the LLNA: DA when using various single alternative decision criteria was evaluated using the traditional LLNA as the reference test. Compared to the traditional LLNA (SI ≥ 3.0), the optimum performance (i.e., accuracy of 93% [41/44] and sensitivity of 100% [32/32]) was achieved using the decision criterion of SI ≥ 1.8 (**Table C-8**). Although the SI ≥ 1.8 produced a false positive rate of 25% (3/12) it yielded a false negative rate of 0% (0/32) (**Table C-8**). Increasing the SI decision criterion to SI ≥ 2.5 decreased the false positive rate to 0% (0/12) but increased the false negative rate to 13% (4/32). The 0% false positive rate using SI ≥ 2.5 and the 0% false negative rate using SI ≥ 1.8 prompted an evaluation using two SI decision criteria for determining LLNA: DA results: one criterion to classify substances as sensitizers (SI ≥ 2.5) and one criterion to classify substances as nonsensitizers (SI ≤ 1.8). This evaluation is described in detail in **Annex VII**.

Figure C-2 Dose Response Curves for Tests Identified as Sensitizers by the LLNA: DA but as Nonsensitizers by the Reduced LLNA: DA



Note: The horizontal line in each figure indicates an SI ≥ 1.8, which is the threshold that is considered optimum for providing a positive response in the LLNA: DA. Points on or above this line would indicate a positive (sensitizer) response, while points below this line would indicate a negative (nonsensitizer) response.

7.0 LLNA: DA Test Method Reliability

An assessment of test method reliability (intralaboratory repeatability and intra- and interlaboratory reproducibility) is an essential element of any evaluation of the performance of an alternative test method (ICCVAM 2003). Repeatability refers to the closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period (ICCVAM 1997, 2003). Intralaboratory reproducibility refers to the extent to which qualified personnel within the same laboratory can replicate results using a specific test protocol at different times. Interlaboratory reproducibility refers to the extent to which different laboratories can replicate results using the same protocol and test substances, and indicates the extent to which a test method can be transferred successfully among laboratories. With regard to the LLNA: DA test method, there are no known intralaboratory repeatability studies, which was also the situation with the traditional LLNA.

The LLNA: DA data were amenable to both intralaboratory and interlaboratory reproducibility analyses. The evaluation of a single decision criterion in **Section 6.5** showed that SI ≥ 1.8 was the SI value that produced the most optimum results (i.e., accuracy of 93% [41/44], sensitivity of 100% [32/32], and false negative rate of 0% [0/32]) among the alternative decision criteria evaluated when the traditional LLNA was the reference test (**Table C-8)**. Thus, this section provides an assessment of reproducibility for the decision criterion of SI ≥ 1.8 to identify sensitizers. For additional reproducibility analyses using a single decision criterion see **Annex VIII,** which describes the evaluation of reproducibility for the decision criterion of SI ≥ 3.0 (SI decision criterion used in the intralaboratory and the interlaboratory validation studies) and SI ≥ 2.0 (previously evaluated as an optimum decision criterion in the March 2009 revised draft BRD evaluated by the Panel) to identify sensitizers. Further, the reproducibility analyses based on the evaluation of multiple decision criteria briefly mentioned in **Section 6.8** (i.e., SI ≥ 2.5 as the decision criterion for classifying substances as sensitizers when used with a decision criterion of SI ≤ 1.8 to identify nonsensitizers) is detailed in **Annex VII**.

7.1 Intralaboratory Reproducibility

Idehara et al. (2008) evaluated intralaboratory reproducibility of EC3 values for the LLNA: DA using two substances (isoeugenol and eugenol) that were each tested in three different experiments (**Table C-13**). The data indicate CV values of 21% and 11% for isoeugenol and eugenol, respectively. The authors state that for both compounds the EC3 values appeared to be close and that for each test substance the SI values for the same concentration were fairly reproducible (Idehara et al. 2008). NICEATM also determined the intralaboratory reproducibility of EC1.8 values (estimated concentration needed to produce an SI of 1.8) for the same set of data. This resulted in CV values of 36% and 23% for isoeugenol and eugenol indicating larger intralaboratory variability compared to EC3 values with CV values of 21% and 11% for isoeugenol and eugenol, respectively.

Table C-13 Intralaboratory Reproducibility of EC3 and EC1.8 Values Using the LLNA: DA[1]

Isoeugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
Vehicle (AOO)	1.00 ± 0.54	1.00 ± 0.54	1.00 ± 0.30
0.5	1.50 ± 0.54	-------	1.22 ± 0.13
1	2.28 ± 0.60	-------	2.77 ± 1.01
2.5	2.78 ± 0.17	3.11 ± 1.15	3.01 ± 0.98

continued

Table C-13 Intralaboratory Reproducibility of EC3 and EC1.8 Values Using the LLNA: DA[1] (continued)

Isoeugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
5	3.39 ± 0.69	4.39 ± 1.25	-------
10	5.68 ± 1.19	6.77 ± 0.23	-------
EC3	**3.40%**	**2.35%**	**2.46%**
EC1.8	**0.69%**	**1.23%**	**0.69%**
Mean EC3: 2.74% ± 0.58% and 21% CV *Mean EC1.8: 0.87% ± 0.31% and 36% CV*			
Eugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
Vehicle (AOO)	1.00 ± 0.17	1.00 ± 0.17	1.00 ± 0.09
5	2.92 ± 1.00	2.80 ± 1.08	3.24 ± 0.70
10	7.35 ± 2.62	4.47 ± 0.98	4.79 ± 0.94
25	10.92 ± 3.63	5.62 ± 3.20	7.07 ± 0.44
EC3	**5.09%**	**5.59%**	**4.50%**
EC1.8	**4.20%**	**3.30%**	**2.63%**
Mean EC3: 5.06% ± 0.55% and 11% CV *Mean EC1.8: 3.38% ± 0.79% and 23% CV*			

Abbreviations: AOO = acetone: olive oil (4:1); CV = coefficient of variation; EC1.8 = estimated concentration needed to produce a stimulation index of 1.8; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] Based on results discussed in Idehara et al. 2008; the number per group was not specified.

[2] Mean stimulation index value ± standard deviation.

7.2 Interlaboratory Reproducibility

Furthermore, data were submitted to NICEATM (**Annex IV**) from a two-phased interlaboratory validation study on the LLNA: DA test method (Omori et al. 2008). In the first phase of the interlaboratory validation study, a blinded test of 12 substances was conducted in 10 laboratories. Three substances (2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, and isopropanol) were tested in all 10 laboratories. The remaining nine substances were randomly assigned to subsets of three of the 10 laboratories **(Table C-14)**. In each laboratory, each substance was tested one time at three different concentrations. The dose levels for each substance were predetermined (i.e., the participating laboratories did not determine their own dose levels for testing). Nine substances are sensitizers and three substances are nonsensitizers according to traditional LLNA results. Six substances are ICCVAM-recommended LLNA performance standards reference substances: cobalt chloride, 2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, isoeugenol, isopropanol, and methyl salicylate.

The second phase of the interlaboratory validation study was designed to evaluate the reliability of the LLNA: DA for testing metallic salts using DMSO as a vehicle since two metals dissolved in DMSO (cobalt chloride and nickel (II) sulfate hexahydrate) from the first phase of the interlaboratory validation study yielded inconsistent results. Five coded substances (two of the five substances were unique to the second phase of the interlaboratory validation study) were tested in seven laboratories

(Table C-15). One substance (i.e. hexyl cinnamic aldehyde) was tested in all seven laboratories. The remaining four substances (cobalt chloride, nickel (II) sulfate hexahydrate, lactic acid, and potassium dichromate) were randomly assigned to subsets of four of the seven laboratories. Each laboratory tested the substance one time at three different dose levels. Again, the dose levels for each substance were predetermined. Of the two substances not previously tested in the first phase of the interlaboratory validation study (lactic acid and potassium dichromate), one is a nonsensitizer and the other is a sensitizer according to traditional LLNA results, respectively. In addition, lactic acid is an ICCVAM-recommended LLNA performance standards reference substance.

The LLNA: DA test results from the two-phased interlaboratory validation study are amenable to interlaboratory reproducibility analyses for three endpoints: sensitizer (positive) or nonsensitizer (negative) classification, and EC1.8 values. Analyses of interlaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer) (**Section 7.2.1**) and a CV analysis for the quantitative results (EC1.8 values) (**Sections 7.2** and **7.3**).

Table C-14 Substances and Allocation for the First Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	Vehicle	Concentration Tested (%)			Laboratory									
					1	2	3	4	5	6	7	8	9	10
2,4-Dinitro-chlorobenzene (+)	AOO	0.03	0.10	0.30	X	X	X	X	X	X	X	X	X	X
Hexyl cinnamic aldehyde (+)	AOO	5	10	25	X	X	X	X	X	X	X	X	X	X
Isopropanol (-)	AOO	10	25	50	X	X	X	X	X	X	X	X	X	X
Abietic acid (+)	AOO	5	10	25		X				X	X			
3-Aminophenol (+)	AOO	1	3	10	X		X					X		
Dimethyl isophthalate (-)	AOO	5	10	25	X		X				X			
Isoeugenol (+)	AOO	1	3	10				X	X				X	
Methyl salicylate (-)	AOO	5	10	25			X				X			X
Formaldehyde (+)	ACE	0.5	1.5	5.0	X	X			X					
Glutaraldehyde (+)	ACE	0.05	0.15	0.50	X	X			X					
Cobalt chloride[2] (+)	DMSO	0.3	1.0	3.0				X		X		X		
Nickel (II) sulfate hexahydrate (+)	DMSO	1	3	10				X		X		X		

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

Table C-15 Substances and Allocation for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	Vehicle	Concentration Tested (%)			Laboratory						
					11	12	13	14	15	16	17
Hexyl cinnamic aldehyde (+)	AOO	5	10	25	X	X	X	X	X	X	X
Cobalt chloride[2] (+)	DMSO	1	3	5	X		X	X			X
Lactic acid (-)	DMSO	5	10	25	X		X		X	X	
Nickel (II) sulfate hexahydrate (+)	DMSO	1	3	10	X	X		X		X	
Potassium dichromate (+)	DMSO	0.1	0.3	1.0	X	X			X		X

Abbreviations: AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

7.2.1 Interlaboratory Reproducibility – Qualitative Results

The qualitative (positive/negative) interlaboratory concordance analysis for the 12 substances that were tested during the first phase of the LLNA: DA interlaboratory validation study is shown in **Table C-16** for SI ≥ 1.8. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), nine substances tested in either three or 10 laboratories had consistent results leading to 100% (3/3 or 10/10) interlaboratory concordance for those substances. There were three substances with discordant results between the labs (isopropanol, 3-aminophenol and nickel [II] sulfate hexahydrate). The interlaboratory concordance for isopropanol was 90% (9/10) and the one discordant lab reported a maximum SI = 1.97 at the lowest dose tested. The interlaboratory concordance for 3-aminophenol and nickel (II) sulfate hexahydrate was 67% (2/3). Two of the three laboratories that tested 3-aminophenol reported SI ≥ 1.8 at the middle dose tested (SI = 2.32 and SI = 1.99 at 10%) and one laboratory did not achieve SI ≥ 1.8 at any dose tested (**Annex IV**). One of the three laboratories that tested nickel (II) sulfate hexahydrate reported a maximum SI = 1.52, while the other two laboratories produced an SI ≥ 1.8 at all three doses tested (**Annex IV**). Notably, when analyzing the dose response curves for the three tests performed for nickel (II) sulfate in the first phase of the two-phased interlaboratory validation study, only one study demonstrated a sufficient dose response (i.e., a parallel increase in SI relative to increase in concentration). Since the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), there were no traditional LLNA concordance data for comparison with the LLNA: DA concordance data from the first phase of the interlaboratory validation study.

Table C-16 Qualitative Results for the First Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 1.8)

Substance Name[1]	Qualitative Results (Maximum SI)[2]										Concordance
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	
2,4-Dinitro-chlorobenzene (+)	+ (11.97)	+ (9.23)	+ (9.96)	+ (8.53)	+ (7.86)	+ (15.14)	+ (13.18)	+ (12.60)	+ (10.89)	+ (4.71)	10/10
Hexyl cinnamic aldehyde (+)	+ (5.78)	+ (4.82)	+ (4.44)	+ (5.11)	+ (3.97)	+ (5.50)	+ (7.09)	+ (10.22)	+ (3.88)	+ (3.51)	10/10
Isopropanol (-)	**- (1.54)**	**- (0.91)**	**- (1.01)**	**- (1.57)**	**- (0.76)**	**+ (1.97)**	**- (1.45)**	**- (1.21)**	**- (0.70)**	**- (1.25)**	**9/10**
Abietic acid (+)		+ (4.64)				+ (7.96)	+ (3.98)				3/3
3-Aminophenol (+)	**+ (2.83)**		**- (1.76)**					**+ (2.38)**			**2/3**
Dimethyl isophthalate (-)	- (1.34)		- (1.29)				- (1.26)				3/3
Isoeugenol (+)				+ (6.11)	+ (5.54)				+ (7.09)		3/3
Methyl salicylate (-)			- (1.55)				- (1.77)			- (0.83)	3/3
Formaldehyde (+)	+ (4.84)	+ (3.18)			+ (2.69)						3/3
Glutaraldehyde (+)	+ (5.00)	+ (3.39)			+ (2.57)						3/3
Cobalt chloride[3] (+)				+[4] (2.66)		+ (20.55)		+ (8.07)			3/3
Nickel (II) sulfate hexahydrate (+)				**-[5] (1.52)**		**+ (11.78)**		**+[5] (3.49)**			**2/3**

Bolded substances did not achieve 100% interlaboratory concordance.

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.

[2] (+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests. Highest stimulation index value for each test is shown in parentheses.

[3] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

[4] Data not reported for the highest dose (3%), only for 0.3% and 1%.

[5] Insufficient dose response.

The qualitative (positive/negative) interlaboratory concordance analysis for the five substances that were tested during the second phase of the LLNA: DA interlaboratory validation study is shown in **Table C-17**. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), four substances (hexyl cinnamic aldehyde, cobalt chloride, lactic acid, and potassium dichromate) tested in either four or seven laboratories had consistent results leading to 100% (4/4 or 7/7) interlaboratory concordance for those substances. There was one discordant substance (nickel [II] sulfate hexahydrate) for which interlaboratory concordance was 75% (3/4). Three of the four laboratories that tested nickel (II) sulfate hexahydrate did not report a maximum SI ≥ 1.8 at any dose, while one laboratory produced an SI ≥ 1.8 at the lowest dose tested. Nickel (II) sulfate hexahydrate was also tested in the first phase of the interlaboratory validation study where interlaboratory concordance was 67% (2/3). Furthermore, as mentioned previously, the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), and therefore there were no traditional LLNA concordance data for comparison with the LLNA: DA concordance data from the second phase of the interlaboratory validation study.

Table C-17 Qualitative Results for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 1.8)

Substance Name[1]	Qualitative Results (Maximum SI)[2]							Concordance
	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17	
Hexyl cinnamic aldehyde (+)	+ (4.47)	+ (5.71)	+ (5.41)	+ (7.60)	+ (3.92)	+ (8.42)	+ (6.45)	7/7
Cobalt chloride[3] (+)	+ (2.01)		+ (2.54)	+ (4.25)			+ (5.06)	4/4
Lactic acid (-)	- (0.93)		- (0.99)		- (0.97)	- (0.91)		4/4
Nickel (II) sulfate hexahydrate (+)	**- (0.79)**	**- (1.24)**		**+ (2.13)**		**- (1.56)**		**3/4**
Potassium dichromate (+)	+ (4.78)	+ (4.08)			+ (6.01)		+ (6.37)	4/4

Bolded substance did not achieve 100% interlaboratory concordance.

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.

[2] (+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests. Highest stimulation index value for each test is shown in parentheses,

[3] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

7.2.2 Interlaboratory Reproducibility – EC1.8 Values

The quantitative (i.e., EC1.8 value) data for interlaboratory reproducibility analysis were obtained from the LLNA: DA results that yielded positive results (SI ≥ 1.8) during the first and second phases of the LLNA: DA interlaboratory validation study. The equation used for calculating EC1.8 values for the positive results was modified based on the method of linear interpolation reported by Gerberick et al. (2004) for the EC3 value:

$$EC1.8 = c + \left[\frac{(1.8 - d)}{(b - d)}\right] \times (a - c)$$

where the data points lying immediately above and below the SI = 1.8 on the dose response curve have the coordinates of (a, b) and (c, d), respectively (Gerberick et al. 2004). For substances for which the lowest concentration tested resulted in an SI ≥ 1.8, an EC1.8 value was extrapolated according to the equation:

$$EC1.8_{ex} = 2^{\left\{\log_2(c) + \frac{(1.8-d)}{(b-d)} \times \left[\log_2(a) - \log_2(c)\right]\right\}}$$

where the point with the higher SI is denoted with the coordinates of (a, b) and the point with the lower SI is denoted (c, d) (Gerberick et al. 2004).

The EC1.8 values from each laboratory were used to calculate CV values for each substance. The resulting values for the first and second phases of the interlaboratory validation study are shown in **Tables C-19** and **C-20**, respectively. In the first phase of the interlaboratory validation study, CV values ranged from 15% (abietic acid) to 140% (isoeugenol) and the mean CV was 71% (**Table C-18**). In the second phase of the interlaboratory validation study, CV values ranged from 14% (hexyl cinnamic aldehyde) to 93% (cobalt chloride) and the mean CV was 49% (**Table C-19**).

The ICCVAM-recommended LLNA performance standards indicate that interlaboratory reproducibility should be evaluated with at least two sensitizing chemicals with well-characterized activity in the traditional LLNA. Acceptable reproducibility is attained when each laboratory obtains ECt values (estimated concentrations needed to produce an SI of a specified threshold) within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20% for hexyl cinnamic aldehyde (ICCVAM 2009). In the first phase of the interlaboratory validation study, eight laboratories reported EC1.8 values outside the acceptance range indicated for 2,4-dinitrochlorobenzene; all of the eight laboratories obtained EC1.8 values that were lower than the specified acceptance range (<0.025%) (**Table C-18**). For hexyl cinnamic aldehyde, all the laboratories participating in the first phase of the interlaboratory validation study obtained an EC1.8 value within the acceptance range (5% to 20%). In the second phase of the interlaboratory validation study, only hexyl cinnamic aldehyde was tested and five of the seven laboratories obtained EC1.8 values that were within the acceptance range indicated (**Table C-19**).

Table C-18 EC1.8 Values from the First Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name	EC1.8 (%)										Mean EC1.8 (%) ± SD	CV (%)
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10		
2,4-Dinitrochlorobenzene (+)	**0.018 (11.97)**	**0.018 (9.23)**	**0.023 (9.96)**	**0.014 (8.53)**	**0.081 (7.86)**	**0.014 (15.14)**	**0.006 (13.18)**	**0.017 (12.60)**	**0.012 (10.89)**	**0.077 (4.71)**	**0.028 ± 0.027**	**97**
Hexyl cinnamic aldehyde (+)	**6.358 (5.78)**	**6.687 (4.82)**	**7.346 (4.44)**	**5.884 (5.11)**	**9.597 (3.97)**	**5.961 (5.50)**	**5.479 (7.09)**	**5.783 (10.22)**	**8.457 (3.88)**	**6.508 (3.51)**	**6.806 ± 1.312**	**19**
Isopropanol (-)	NA	NA	NA	NA	NA	IDR	NA	NA	NA	NA	NA	NA
Abietic acid (+)		3.636				4.878	4.598				4.371 ± 0.651	15
3-Aminophenol (+)	1.175		NA					2.507			1.841 ± 0.942	51
Dimethyl isophthalate (-)	NA		NA				NA				NA	NA
Isoeugenol (+)				0.337	4.082				0.265		1.561 ± 2.183	140
Methyl salicylate (-)			NA				NA			NA	NA	NA
Formaldehyde (+)	0.209	0.579			1.380						0.723 ± 0.599	83
Glutaraldehyde (+)	0.064	0.235			0.104						0.134 ± 0.089	67
Cobalt chloride[2] (+)				0.233[3]		0.025		0.071			0.110 ± 0.109	99
Nickel (II) sulfate hexahydrate (+)				NA		0.188		IDR			0.188 ± NA	NA

Bolded text indicates substances that are ICCVAM-recommended murine local lymph node assay (LLNA) performance standards reference substances for evaluating interlaboratory reproducibility (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For both 2,4-dinitrochlorobenzene and hexyl cinnamic aldehyde, the highest SI values achieved were from the highest dose tested (0.3% for 2,4-dinitrochlorobenzene and 25% for hexyl cinnamic aldehyde). Shading shows EC1.8 values that are outside of the acceptable range indicated in the ICCVAM-recommended LLNA performance standards: 5-20% for hexyl cinnamic aldehyde and 0.025-0.1% for 2,4-dinitrochlorobenzene.

Abbreviations: CV = coefficient of variation; EC1.8 = estimated concentration needed to produce a stimulation index of 1.8; IDR = insufficient dose response for calculation of EC1.8; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; NA = not applicable; SD = standard deviation.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

[3] Data not reported for the highest dose (3%), only for 0.3% and 1%.

Table C-19 EC1.8 Values from the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	EC1.8 (%)							Mean EC1.8 (%) ± SD	CV (%)
	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17		
Hexyl cinnamic aldehyde (+)	**5.793 (4.47)**	**5.426 (5.71)**	**5.627 (5.41)**	**4.442 (7.60)**	**6.469 (3.92)**	**4.437 (8.42)**	**5.720 (6.45)**	**5.416 ± 0.741**	**14**
Cobalt chloride[2] (+)	3.499		1.382	0.723			0.393	1.499 ± 1.395	93
Lactic acid (-)	NA		NA		NA	NA		NA	NA
Nickel (II) sulfate hexahydrate (+)	NA	NA		5.938		NA		5.938 ± NA	NA
Potassium dichromate (+)	0.089	0.089			0.046		0.041	0.066 ± 0.026	39

Bolded text indicates a substance that is an ICCVAM-recommended murine local lymph node assay (LLNA) performance standards reference substance for evaluating interlaboratory reproducibility (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For hexyl cinnamic aldehyde, the highest SI values achieved were from the highest dose tested (25%). Two of the EC1.8 values (shaded cells) are outside of the acceptable range indicated in the ICCVAM-recommended LLNA performance standards (5-20% for hexyl cinnamic aldehyde).

Abbreviations: CV = coefficient of variation; EC1.8 = estimated concentrations needed to produce a stimulation index of 1.8; NA = not applicable; SD = standard deviation.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

The interlaboratory CV values for both the first and second phases of the interlaboratory validation study for the LLNA: DA EC1.8 values were higher than that for the traditional LLNA EC3 values. The analysis of interlaboratory variation of EC3 values for the traditional LLNA reported CV values of 6.8% to 83.7% for five substances tested in five laboratories (**Table C-20**; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: DA (hexyl cinnamic aldehyde, 2,4-dinitrochlorobenzene, and isoeugenol). All interlaboratory CV values for the LLNA: DA were greater than that for the traditional LLNA. The CV of 97% for 2,4-dinitrochlorobenzene was greater than the two CV values of 37.4% and 27.2% (which were calculated from five values each), reported by ICCVAM (1999). The CV of 19% and 14% for hexyl cinnamic aldehyde tested in the first and second phases of the LLNA: DA interlaboratory validation study, respectively, were both greater than the 6.8% reported by ICCVAM (1999). The CV of 140% for isoeugenol tested in the LLNA: DA was greater than the 41.2% reported by ICCVAM (1999).

Table C-20 Interlaboratory Reproducibility of the EC3 Values for Substances Tested in the Traditional LLNA[1]

Substance Name	EC3 (%)					CV (%)
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	
2, 4-Dinitrochlorobenzene	0.3	0.5	0.6	0.9	0.6	37.4
	0.5	0.6	0.4	0.6	0.3	27.2
Hexyl cinnamic aldehyde	7.9	7.6	8.4	7.0	8.1	6.8
Isoeugenol	1.3	3.3	1.8	3.1	1.6	41.2
Eugenol	5.8	14.5	8.9	13.8	6.0	42.5
SLS	13.4	4.4	1.5	17.1	4.0	83.7

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA = murine local lymph node assay; SLS = sodium lauryl sulfate.

[1] From ICCVAM 1999 report.

7.3 Reproducibility Analysis for Substances with Multiple Tests

Section 6.5 details the accuracy analysis for the LLNA: DA (using the most prevalent outcome for substances with multiple tests) when using one optimized criterion to classify substances as potential sensitizers (SI ≥ 1.8). SI ≥ 1.8 was evaluated for classifying substances as potential sensitizers because it resulted in no false negative results, with respect to traditional LLNA data. This section examines the reproducibility of the tests for the 14 substances that had multiple LLNA: DA test results, regardless of whether the tests were performed in one laboratory or multiple laboratories. The frequency with which SI values for the 14 substances occurred in one of three SI categories was considered. The three SI categories were:

- LLNA: DA nonsensitizers with SI < 1.8
- LLNA: DA sensitizers with SI between 1.8 and 2.5 (borderline positive results with potential to be false positives with respect to classification by the traditional LLNA)
- LLNA: DA sensitizers with SI ≥ 2.5

For the 14 substances, three to 18 tests were available. **Table C-21** shows the proportion of the tests for each substance that produced SI values in each category. For the four traditional LLNA nonsensitizers with multiple test results, there were 23 LLNA: DA tests that produced SI < 1.8 and one LLNA: DA test that produced an SI between 1.8 and 2.5. For the 10 traditional LLNA sensitizers with multiple LLNA: DA test results, however, SI values occurred in all three SI categories. The results for nickel (II) sulfate hexahydrate were particularly variable: 50% (4/8) produced SI < 1.8 (four tests with SI = 0.79, 1.24, 1.52, and 1.56), 25% (2/8) produced 1.8 < SI < 2.5 (SI = 2.13 and 2.17), and 25% (2/8) produced SI ≥ 2.5 (SI = 3.49 and 11.78). 3-Aminophenol also produced SI values in all three categories: 33% (1/3) of the tests had SI < 1.8 (SI = 1.76), 33% (1/3) of the tests had 1.8 < SI < 2.5 (SI = 2.38), and 33% (1/3) of the tests had SI ≥ 2.5 (SI = 2.83). Cobalt chloride tests produced SI values in two categories: 12.5% (1/8) of the tests had 1.8 < SI < 2.5 (SI = 2.01) and seven of eight tests (87.5%) produced SI ≥ 2.5 (SI = 2.54, 2.66, 3.64, 4.25, 5.06, 8.07, and 20.55). The multiple test results for the remaining seven traditional LLNA sensitizers were 100% concordant (**Table C-21**).

Table C-21 Concordance of LLNA: DA Tests for Substances with Multiple Tests by Maximum SI Category

Substance Name	LLNA: DA Nonsensitizers (Maximum SI < 1.8)[1]	LLNA: DA Sensitizers (SI ≥ 1.8)		
		1.8 < Maximum SI < 2.5[1]	Maximum SI ≥ 2.5[1]	Total Tests
Sensitizers[2]				
Abietic acid	0 (0%)	0 (0%)	4 (100%)	4
3-Aminophenol	1 (33.3%)	1 (33.3%)	1 (33.3%)	3
Cobalt chloride	0 (0%)	1 (12.5%)	7 (87.5%)	8
2,4-Dinitrochlorobenzene	0 (0%)	0 (0%)	11 (100%)	11
Formaldehyde	0 (0%)	0 (0%)	4 (100%)	4
Glutaraldehyde	0 (0%)	0 (0%)	4 (100%)	4
Hexyl cinnamic aldehyde	0 (0%)	0 (0%)	18 (100%)	18
Isoeugenol	0 (0%)	0 (0%)	4 (100%)	4
Nickel (II) sulfate hexahydrate	4 (50%)	2 (25%)	2 (25%)	8
Potassium dichromate	0 (0%)	0 (0%)	5 (100%)	5
Nonsensitizers[2]				
Dimethyl isophthalate	4 (100%)	0 (0%)	0 (0%)	4
Isopropanol	10 (91%)	1 (9%)	0 (0%)	11
Lactic acid	5 (100%)	0 (0%)	0 (0%)	5
Methyl salicylate	4 (100%)	0 (0%)	0 (0%)	4

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] Numbers shown reflect number of tests. Percentage in parentheses reflects percentage of the total number of tests for each substance.

[2] According to traditional LLNA results.

8.0 LLNA: DA Data Quality

All of the studies included in this performance evaluation are based on individual animal data submitted to NICEATM in the form of original data and study records. Furthermore, manuscripts detailing the results for 31 substances evaluated in the intralaboratory study and 14 substances evaluated in the two-phased interlaboratory validation have been published in the peer-reviewed literature (Idehara et al. 2008; Omori et al. 2008). An independent audit has been conducted to confirm that the reported data from the intralaboratory validation study (assessment of 31 substances from Idehara et al. 2008) performed by Daicel Chemical Industries, Ltd. were the same as the data originally recorded (Idehara et al. 2008). The data from the two-phased interlaboratory validation study were not subjected to a formal audit, but the raw data were reportedly entered directly into formatted MS-Excel templates provided by the study management team prior to being used for analyses (Omori et al. 2007). Data recently received for 14 substances evaluated in an intralaboratory validation study (Idehara unpublished) were also not subjected to a formal audit. The intralaboratory assessment at Daicel Chemical Industries, Ltd. (Idehara et al. 2008; Idehara unpublished), as well as the two-phased interlaboratory validation study (Omori et al. 2008), did not conduct their studies in compliance with Good Laboratory Practice guidelines, although all of the participating laboratories reportedly have this capability.

9.0 Other Scientific Reports and Reviews

Yamashita et al. (2005) describe the development of the LLNA: DA as an alternative nonradioisotope LLNA test method. The manuscript details the determination of an optimal dosing schedule and further compares SI values obtained from lymph node weights versus ATP content to determine an appropriate lymphocyte proliferation endpoint. The authors further assess the intermediate precision and sensitivity/specificity of the LLNA: DA. In those experiments, four compounds (2,4-dinitrochlorobenzene, eugenol, α-hexyl cinnamic aldehyde, and methyl salicylate) were tested and no significant differences were noted in the SI levels generated from the LLNA: DA and the traditional LLNA. The studies by Yamashita et al. provided the basis for the expanded intralaboratory study of 31 substances performed by Daicel Chemical Industries, Ltd. and published by Idehara et al. (2008) (described in **Sections 6.0** and **7.0**).

Idehara et al. (2008) summarize the LLNA: DA test method in terms of test substance dosing schedule, preparation of single cell suspensions of the auricular lymph nodes, measurement of ATP content, and explanation of statistical analyses employed. The authors further describe how the results correlate between ATP content and lymph node cell number, the test results (i.e., mean SI values and EC3 values) obtained for the 31 substances, the concordance of the LLNA: DA versus the traditional LLNA EC3 values, and the reproducibility of EC3 and SI values. Based on the details included in the manuscript, the authors conclude that the SI values obtained from measuring ATP content were similar to the traditional LLNA and therefore the LLNA: DA was a promising nonradioisotope modified test method for evaluating the skin sensitization potential of substances.

Omori et al. (2008) describe the two-phased interlaboratory validation study used to evaluate the reliability and relevance of the LLNA: DA test method (see **Section 7.0**). They describe the organization and technology transfer of the test method between the laboratories, as well as test substance selection and allocation. They further describe the development of the LLNA: DA and the resulting standard protocol for the LLNA: DA interlaboratory study. They provide the interlaboratory data for analyzing both ATP content with regard to SI values and lymph node weight and discuss assay sensitivity and interlaboratory variability. Based on the data summarized in the manuscript, the authors conclude that in the first phase of the interlaboratory validation study, a large variation was observed for two substances (cobalt chloride and nickel [II] sulfate hexahydrate) but in the second phase of the interlaboratory validation study this variation was small. The authors attribute the initial variation to application of DMSO as the solvent for the metallic salts and therefore, prior to the second phase of the interlaboratory validation study, include operation of LLNA: DA with DMSO in the technology transfer seminar. In conclusion, the authors view the LLNA: DA as a reliable test method for predicting skin sensitization potential of substances.

Regarding the LLNA: DA test method, noncommission members of JaCVAM met on August 28, 2008 at the National Institute of Health Sciences, Tokyo, Japan, and endorsed the following statement: “Following the review of the results of the Ministry of Health, Labour and Welfare (MHLW)-funded validation study on the LLNA: DA coordinated by Japanese Society for Alternative to Animal Experiments, it is concluded that the LLNA: DA can be used for distinguishing between sensitizer and nonsensitizer chemicals within the context of the OECD testing guidelines No. 429 on skin sensitization: LLNA. The JaCVAM regulatory acceptance board has been regularly kept informed of the progress of the study, and this endorsement was based on an assessment of various documents, including, in particular, the report on the results from the study, and also on the evaluation supported by MHLW of the study prepared for the JaCVAM ad hoc peer review panel.”

10.0 Animal Welfare Considerations

The LLNA: DA will require the use of the same number of animals when compared to the updated ICCVAM-recommended LLNA protocol (Appendix A of ICCVAM 2009). However, since the traditional LLNA uses radioactive materials and as such its use might be restricted in some countries and institutions due to the complications associated with storage, use, and disposal, broader use of a nonradioactive alternative to the traditional LLNA, such as the LLNA: DA, could further reduce the number of GPs that are used to assess skin sensitization.

Further, the LLNA: DA offers increased refinement by avoiding the discomfort that can occur in the guinea pig tests when substances cause ACD. Additionally, the LLNA: DA test method protocol requires fewer mice per treatment group (a minimum of four animals per group) than either of the guinea pig tests (10-20 animals/group for the Buehler test and 5-10 animals/group for the GPMT).

10.1 Rationale for the Need to Use Animals

The rationale for the use of animals in the LLNA: DA is the same as the rationale for the traditional LLNA. There currently are no valid and accepted non-animal test methods to determine the ACD potential of substances and products, except for situations where human studies could be conducted ethically and where such studies would meet regulatory safety assessment requirements. Additionally, the most detailed information about the induction and regulation of immunological responses are available for mice (ICCVAM 1999).

10.2 Basis for Determining the Number of Animals Used

The number of animals used for the experimental, vehicle, and positive control groups is based on the number of animals used in the development (Yamashita et al. 2005) and validation of the test method (Idehara et al. 2008; Omori et al. 2008), which is the same as that specified in the updated ICCVAM-recommended LLNA protocol (Appendix A of ICCVAM 2009).

10.3 Reduction Considerations

A further reduction of up to 40% (15 vs. 25) could be achieved by using a reduced version of the LLNA: DA, in cases where dose-response information is not needed for hazard identification purposes. In such an approach, only the highest dose of the test article that does not elicit excessive skin irritation or systemic toxicity would be administered, and the two lower dose groups would not be used. Additional reductions could be achieved by testing more substances concurrently, so that the same vehicle and positive control group could be used for multiple substances.

11.0 Practical Considerations

Several issues are taken into account when assessing the practicality of using an alternative to an existing test method. In addition to performance evaluations, assessments of the laboratory equipment and supplies needed to conduct the alternative test method, level of personnel training, labor costs, and the time required to complete the test method relative to the existing test method are necessary. The time, personnel cost, and effort required to conduct the proposed test method(s) must be considered to be reasonable when compared to the existing test method it is intended to replace.

11.1 Transferability of the LLNA: DA

Test method transferability addresses the ability of a method to be accurately and reliably performed by multiple laboratories (ICCVAM 2003), including those experienced in the particular type of procedure as well as laboratories with less or no experience in the particular procedure. It would be expected that the transferability of the LLNA: DA would be similar to the traditional LLNA, since their test method protocols are experimentally similar. Notably, the test method developer does indicate that when the LLNA: DA test method is conducted, all the procedural steps from lymph node excision to the determination of ATP content should be performed without delay since ATP content decreases over time (Idehara et al. 2008; Omori et al. 2008). The first and second phases of the interlaboratory validation study have demonstrated that this test method is transferable (see **Section 7.0**).

11.2 Laboratories and Major Fixed Equipment Required to Conduct the LLNA: DA

Compared to the traditional LLNA, the LLNA: DA will not require laboratories, equipment, and licensing permits for handling radioactive materials. However, the LLNA: DA does require access to a luminometer capable of detecting light emission by ATP for the assessment of lymphocyte proliferation. The remaining requirements (e.g., animal care laboratories) are the same between the two methods.

11.3 LLNA: DA Training Considerations

The level of training and expertise needed to conduct the LLNA: DA should be similar to the traditional LLNA, although the LLNA: DA includes an additional requirement that users operate a luminometer instead of a scintillation counter and be able to process this data.

12.0 References

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13.0 Glossary

Accuracy:[12] (a) The closeness of agreement between a test method result and an accepted reference value. (b) The proportion of correct outcomes of a test method. It is a measure of test method performance and one aspect of *relevance*. The term is often used interchangeably with *concordance* (see also *two-by-two table*). Accuracy is highly dependent on the prevalence of positives in the population being examined.

Allergic Contact Dermatitis (ACD): A Type IV allergic reaction of the skin that results from repeated skin contact with a skin sensitizer. Clinical signs of ACD include the development of erythema (redness) and edema (swelling), blistering, and itching. Also referred to as skin sensitization.

Assay:[12] The experimental system used. Often used interchangeably with *test* and *test method*.

Coded substances: Substances labeled by code rather than name so that they can be tested and evaluated without knowledge of their identity or anticipation of test results. Coded substances are used to avoid intentional or unintentional bias when evaluating laboratory or test method performance.

Concordance:[12] The proportion of all substances tested that are correctly classified as positive or negative. It is a measure of test method performance and one aspect of *relevance*. The term is often used interchangeably with *accuracy* (see also *two-by-two table*). Concordance is highly dependent on the prevalence of positives in the population being examined.

EC1.8: The estimated concentration needed to produce a stimulation index of 1.8, as compared to the concurrent vehicle control.

EC3: The estimated concentration needed to produce a stimulation index of three, as compared to the concurrent vehicle control.

ECt: The estimated concentration needed to produce a stimulation index of a specific threshold, as compared to the concurrent vehicle control.

False negative:[12] A substance incorrectly identified as negative by a test method.

False negative rate:[12] The proportion of all positive substances falsely identified by a test method as negative (see *two-by-two table*). It is one indicator of test method accuracy.

False positive:[12] A substance incorrectly identified as positive by a test method.

False positive rate:[12] The proportion of all negative substances that are falsely identified by a test method as positive (see *two-by-two table*). It is one indicator of test method accuracy.

Good Laboratory Practices (GLP):[12] Regulations promulgated by the U.S. Food and Drug Administration and the U.S. Environmental Protection Agency, and principles and procedures adopted by the Organisation for Economic Co-operation and Development (OECD) and Japanese authorities, that describe record keeping and quality assurance procedures for laboratory records that will be the basis for data submissions to national regulatory agencies.

Hazard[12]**:** The potential for an adverse health or ecological effect. A hazard potential results only if an exposure occurs that leads to the possibility of an adverse effect being manifested.

Interlaboratory reproducibility:[12] A measure of whether different qualified laboratories using the same protocol and test substances can produce qualitatively and quantitatively similar results.

[12] Definition used by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM 2003).

Interlaboratory reproducibility is determined during the prevalidation and validation processes and indicates the extent to which a test method can be transferred successfully among laboratories.

Intralaboratory repeatability:[12] The closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period.

Intralaboratory reproducibility:[12] The first stage of validation; a determination of whether qualified people within the same laboratory can successfully replicate results using a specific test protocol at different times.

Immunological: Relating to the immune system and immune responses.

In vivo: In the living organism. Refers to assays performed in multicellular organisms.

Lymphocyte: A white blood cell found in the blood, lymph, and lymphoid tissues, which regulates and plays a role in acquired immunity.

Murine local lymph node assay (LLNA): An *in vivo* test method used to assess the skin sensitization potential of a substance by measuring the proliferation of lymphocytes in the lymph nodes draining the ears (i.e., auricular lymph nodes) of mice, subsequent to topical exposure on the ear to the substance. The traditional LLNA measures lymphocyte proliferation by quantifying the amount of ^{3}H-thymidine or ^{125}I-iododeoxyuridine incorporated into the cells of the draining lymph nodes.

Murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content (LLNA: DA): An *in vivo* test method used to assess the skin sensitization potential of a substance by measuring the proliferation of lymphocytes in the lymph nodes draining the ears (i.e., auricular lymph nodes) of mice, subsequent to topical exposure on the ear to the substance. The LLNA: DA is a nonradioactive modification of the traditional LLNA and assesses lymphocyte cell proliferation by measuring increases in ATP content in the lymph node as an indicator of the cell number at the end of cell proliferation.

Negative predictivity:[12] The proportion of correct negative responses among substances testing negative by a test method (see *two-by-two table*). It is one indicator of test method accuracy. Negative predictivity is a function of the sensitivity of the test method and the prevalence of negatives among the substances tested.

Nonsensitizer: A substance that does not cause skin sensitization following repeated skin contact.

Performance:[12] The accuracy and reliability characteristics of a test method (see *accuracy*, *reliability*).

Positive control: A substance known to induce a positive response, which is used to demonstrate the sensitivity of the test method and to allow for an assessment of variability in the conduct of the assay over time. For most test methods, the positive control substance is tested concurrently with the test substance and the vehicle/solvent control. However, for some *in vivo* test methods, periodic studies using a positive control substance are considered adequate by the OECD.

Positive predictivity:[12] The proportion of correct positive responses among substances testing positive by a test method (see *two-by-two table*). It is one indicator of test method accuracy. Positive predictivity is a function of the sensitivity of the test method and the prevalence of positives among the substances tested.

Prevalence:[12] The proportion of positives in the population of substances tested (see *two-by-two table*).

Protocol:[12] The precise, step-by-step description of a test, including the listing of all necessary reagents, criteria and procedures for the evaluation of the test data.

Quality assurance:[12] A management process by which adherence to laboratory testing standards, requirements, and record keeping procedures is assessed independently by individuals other than those performing the testing.

Reduction alternative:[12] A new or modified test method that reduces the number of animals required.

Reference test method:[12] The accepted *in vivo* test method used for regulatory purposes to evaluate the potential of a test substance to be hazardous to the species of interest.

Refinement alternative:[12] A new or modified test method that refines procedures to lessen or eliminate pain or distress in animals or enhances animal well-being.

Relevance:[12] The extent to which a test method correctly predicts or measures the biological effect of interest in humans or another species of interest. Relevance incorporates consideration of the *accuracy* or *concordance* of a test method.

Reliability:[12] A measure of the degree to which a test method can be performed reproducibly within and among laboratories over time. It is assessed by calculating intra- and interlaboratory reproducibility and intralaboratory repeatability.

Replacement alternative:[12] A new or modified test method that replaces animals with non-animal systems or one animal species with a phylogenetically lower one (e.g., a mammal with an invertebrate).

Reproducibility:[12] The consistency of individual test results obtained in a single laboratory (intralaboratory reproducibility) or in different laboratories (interlaboratory reproducibility) using the same protocol and test substances (see intra- and interlaboratory reproducibility).

rLLNA: DA (reduced LLNA: DA): A variant of the LLNA: DA that employs a single, high dose of the test substance rather than multiple doses to determine its skin sensitization potential, thus using fewer animals.

Sensitivity:[12] The proportion of all positive substances that are classified correctly as positive in a test method. It is a measure of test method accuracy (see *two-by-two table*).

Skin sensitizer: A substance that induces an allergic response following skin contact.

Specificity:[12] The proportion of all negative substances that are classified correctly as negative in a test method. It is a measure of test method accuracy (see *two-by-two table*).

Stimulation index (SI): A value calculated for the LLNA: DA to assess the skin sensitization potential of a test substance. The value is calculated as the ratio of the mean ATP content of the auricular lymph nodes from a group of treated mice to the mean ATP content of the auricular lymph nodes from a group of vehicle control mice. The mean ATP content is measured in relative luminescence units. For the LLNA: DA and the rLLNA: DA, an SI $\geq$ 1.8 classifies a substance as a potential skin sensitizer.

Test:[12] The experimental system used; used interchangeably with *test method* and *assay*.

Test method:[12] A process or procedure used to obtain information on the characteristics of a substance or agent. Toxicological test methods generate information regarding the ability of a substance or agent to produce a specified biological effect under specified conditions. Used interchangeably with *test* and *assay*. See also *validated test method* and *reference test*.

Transferability:[12] The ability of a test method or procedure to be accurately and reliably performed in different, competent laboratories.

Two-by-two table:[12] The two-by-two table can be used for calculating accuracy (concordance) ([a + d]/[a + b+ c + d]), negative predictivity (d/[c + d]), positive predictivity (a/[a + b]), prevalence ([a + c]/[a + b + c + d]), sensitivity (a/[a + c]), specificity (d/[b + d]), false positive rate (b/[b + d]), and false negative rate (c/[a + c]).

		New Test Outcome		
		Positive	**Negative**	**Total**
Reference Test Outcome	**Positive**	a	c	a + c
	Negative	b	d	b + d
	Total	a + b	c + d	a + b + c + d

Validated test method:[12] An accepted test method for which validation studies have been completed to determine the relevance and reliability of this method for a specific proposed use.

Validation:[12] The process by which the reliability and relevance of a procedure are established for a specific purpose.

Vehicle control: An untreated sample containing all components of a test system, including the vehicle that is processed with the test substance-treated and other control samples to establish the baseline response for the samples treated with the test substance dissolved in the same vehicle.

Weight-of-evidence (process): The strengths and weaknesses of a collection of information are used as the basis for a conclusion that may not be evident from the individual data.

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Annex I

LLNA: DA Test Method Protocol

Annex I-1
Standard Operating Procedures Used for the LLNA: DA Test Method Validation Studies .. C-89

Annex I-2
LLNA: DA Test Method Data Comparing With and Without 1% SLS Pretreatment C-105

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Annex I-1

Standard Operating Procedures Used for the LLNA: DA Test Method Validation Studies

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1.0 Introduction

These are the standard operating procedures for the two-phased interlaboratory test method validation study (Omori et al. 2008) for the murine local lymph node assay (LLNA) modified by Daicel Chemical Industries, Ltd., based on ATP content (referred to hereafter as the "LLNA: DA") as confirmed by the LLNA: DA Validation Committee and provided by the study director.[1] These procedures are intended for tests conducted to evaluate a single test substance. Although the standard operating procedures detailed herein are specific for the two-phased interlaboratory test method validation study (Omori et al. 2008), the substances tested in the intralaboratory validation study followed a technically similar LLNA: DA test method protocol (Idehara et al. 2008; Idehara unpublished).

2.0 Preparation of Equipment and Materials

Prepare the experimental equipment, materials, and reagents given in **Table C-I-1**. Luminometer tubes, 15 mL test tubes, 50 mL test tubes, petri dishes, and slide glass should be disposable. The underlined items will be provided by the LLNA: DA Validation Committee but in some cases, a luminometer will be furnished by the test facilities. All other materials will be provided by the test facilities.

Table C-I-1 List of Required Equipment, Materials and Reagents

Name of Equipment, Material, or Reagent	Manufacturer	Comment (Trade Name, Model Number, etc.)
Luminometer	Kikkoman Corporation, Japan	LUMITESTER C-100 Detection Range: $4x10^{-12}$ – $1x10^{-6}$ M Upper Limit: 1,000,000 RLU
Luminometer tubes	Kikkoman Corporation, Japan	Polypropylene, sterilized
15 mL test tubes	IWAKI brand	Polypropylene, sterilized
50 mL test tubes	IWAKI brand	Polypropylene, sterilized
Petri dish	Corning Incorporated	Cell culture dish, sterilized
Cell scraper	Costar brand	Disposable cell scraper, sterilized
Slide glass	Matsunami	Micro slide glass
Vortex mixer		
Analytical balance		For body weight measurements (readability of at least 0.1 g)
Analytical balance		For lymph node weight measurements (readability of at least 0.1 mg)
Brush	Ikkyuen	Osho
Phosphate buffered saline	Invitrogen Gibco™	pH 7.2, sterilized
Luciferin-luciferase reagent	Kikkoman Corporation, Japan	CheckLite™ 250 Plus[1]

continued

[1] Confirmed by LLNA: DA Validation Committee on 2/6/2006; Revised by Takashi Omori, Study Director on: 2/17/2006, 2/19/2006, 3/27/2006, 4/2/2006, and 12/2/2006.

Table C-I-1 List of Required Equipment, Materials and Reagents (continued)

Name of Equipment, Material, or Reagent	Manufacturer	Comment (Trade Name, Model Number, etc.)
Cages		Capable of housing four mice, with feed and water dispensers
Micropipette		For applying test solutions (25 μL), handling phosphate buffered saline (1000 μL), tissue suspension (20 μL), cell suspension (100 μL), and dissolved luciferin-luciferase solution (100 μL)
Micropipette tips		Sterilized
Dissecting instruments		Large and small tweezers, scissors, surgical holder, injection needle and holder
Timer		With second display
General laboratory materials		Cotton, antiseptic solution, paper towel, clean sheet, test tube rack, microtube rack

Abbreviations: RLU = relative luminescence units.

[1] For the intralaboratory validation study by Daicel Chemical Industries, Ltd. (Idehara et al. 2008; Idehara unpublished), only the ATP content for potassium dichromate was measured by the CheckLite™ 250 Plus Kit (Kikkoman Corporation, Japan) and the ViaLight® HS Kit (Lonza Rockland, Inc., USA) was used for determining the ATP content of all the other substances in the intralaboratory validation.

3.0 Preparations Prior to Delivery of Animals

The animals to be used in the tests are young adult female mice (nulliparous and non-pregnant) of the CBA/JNCrlj strain, aged between 8-12 weeks prior to application of test and control substances. The animals will be provided by the LLNA: DA Validation Committee. Preparations should be made according to the standards of the test facilities to begin acclimatizing the animals once they have arrived on the previously agreed upon date of delivery.

Six cages capable of holding four animals each should be prepared prior to the end of acclimatization. The cages should be labeled as listed in **Table C-I-2**. The symbol "X" represents the code of the test substance to be provided. Mark the label using the letter indicated on the datasheets provided prior to the test. The animal test group numbers are also indicated on the datasheets. The numbers should be confirmed and the cages labeled with care. This test will be performed two or three times, so it is important to include the test number on the labels.

Table C-I-2 Preparation of Test Group Cages

Test Group Number	Label
Group 1	Acetone: Olive Oil (4:1)
Group 2	Positive Control
Group 3	Vehicle
Group 4	Test Substance "X" – Low Concentration
Group 5	Test Substance "X" – Medium Concentration
Group 6	Test Substance "X" – High Concentration

"X" represents the code of the test substance provided by the study management team.

4.0 Delivery, Acclimatization and Animal Assignment

On the date of delivery, 25 animals will arrive and acclimatization should begin immediately. Acclimatization should be performed according to the standards of the test facilities. The animals should be acclimatized for at least five days, but no more than 16 days.

After acclimatization healthy animals with no observable skin lesions or other abnormalities should be randomly assigned to six groups of four[2] animals each using randomly generated numbers. After assigning the animals to groups, four animals each should be placed in the six cages prepared as described in **Section 3.0**. Any animals remaining after the assignment of 24 should be omitted from the test. Should there be fewer than 24 animals with no observed abnormalities, three animals should be assigned to each group beginning with the test group with the highest number until all of the animals are assigned.

From the delivery of the animals to the end of the test procedures the temperature of the animal housing facility should be maintained at 22ºC (±3ºC) with a relative humidity of 30-70%. The animals should be housed with a light: dark cycle of 12 hours light: 12 hours dark and should be given food and water *ad libitum*. Any deviations from the standard housing and feeding procedures should be recorded.

5.0 Confirmation of Test Materials

Upon arrival of the test materials, sent by the LLNA: DA Validation Committee, confirm that the inventory document matches the contents.

The labels for each of the treatments (acetone: olive oil [4:1], positive control, vehicle, and low, medium and high concentrations of test substances) include a test substance code and a group number. After confirming that these codes match the datasheet, arrange the treatments in a test tube rack according to group number. Sodium lauryl sulfate (SLS) solution will arrive in one tube. Apportion 3 mL of SLS solution to each of the accompanying empty test tubes, mark each tube with the group number, and arrange the tubes in order in the test tube rack.

The treatments should be refrigerated immediately and only removed when beginning the test. Refrigeration of the solutions used in these procedures should be between 0-10ºC, and preferably between 2-8ºC, except when instructed differently. Should there be specific instructions as to the handling of the solutions, the instructions will be included with the materials shipment and they should be followed. For instance:

- SLS (CASRN: 151-21-3) is a 1% aqueous solution and should be kept at room temperature
- Acetone: olive oil is 4:1 volume to volume ratio
- Positive control is a 25% acetone: olive oil (4:1) solution of hexyl cinnamic aldehyde (CASRN: 101-86-0)[3]

[2] For the tests conducted as part of the intralaboratory validation study by Daicel Chemical Industries, Ltd. (Idehara et al. 2008; Idehara unpublished), at least three animals per dose group were used (i.e., in most cases, four animals per control group and three animals per treatment group).

[3] For the tests conducted as part of the intralaboratory validation study by Daicel Chemical Industries, Ltd., either 15% hexyl cinnamic aldehyde (CASRN: 101-86-0), 10% eugenol (CASRN: 101-86-0), or 5% cinnamic aldehyde (CASRN: 104-55-2) were used as positive controls (Idehara et al. 2008).

6.0 Procedures on Test Days 1, 2, 3 and 7

6.1 Day 1

Mark the animals on the tail with their test group number and a number from 1-4. Weigh the animals and record their weight to the nearest 0.1 g on the test forms.

Remove the test materials from the refrigerator. Should the materials arrive with instructions to heat or sonicate the treatments prior to application, perform these procedures as instructed.

6.1.1 Pre-treatment with 1% SLS Aqueous Solution

Beginning with Group 1 and proceeding in order to Group 6, the SLS solution should be applied with a brush to the dorsum of both ears of the mice. The number of the SLS solution used should match the test group number. The brush should be dipped in the SLS solution and applied to the dorsum of one ear using a petting motion, covering the entire dorsum with four to five strokes. Dip the brush again in the SLS solution and apply the solution to the dorsum of the other ear in the same manner.

Record the time when beginning to apply SLS solution to Group 1 and when completing application to Group 6. The application procedure should be performed continuously without delay for Groups 1-6.

Six brushes should be prepared and numbered, using only one brush for each test group. When performing the same application procedure on Days 2, 3, and 7 there is the possibility of brush contamination due to residual solution on the mouse auricula. It is important to switch brushes after finishing application for one group and check the number of the next brush before proceeding to the next group. After use, the brushes should be washed thoroughly and made available for the next day.

6.1.2 Test Substance Application

One hour after starting the SLS solution application, the numbered treatments should be applied to the auriculae of the mice, beginning with Group 1 and ending with Group 6. Using a micropipette or similar device, 25 μL of the test solution should be dripped slowly on the dorsum of one of the mouse's ears, covering the dorsum entirely. Again take up 25 μL of treatment solution and apply it in the same manner to the dorsum of the mouse's other ear.

When applying the treatments, micropipette tips should be changed for each test group. After completing application for one test group, remove the tip and spray the end of the micropipette with an alcohol mist and wipe to avoid contamination.

Record the time when beginning to apply the test solution to Group 1 and when completing application to Group 6. The application procedure should be performed continuously without delay for Groups 1-6.

Immediately after completing application the test materials should be refrigerated.

6.1.3 General Information on the 1% SLS Pre-treatment and Test Substance Application

The objective of the application procedure is to first apply SLS solution to the entirety of the dorsum of the ear and then to apply a prescribed amount of test solution to the same area. Using ether anesthesia ensures ease and accuracy of the procedure. However, special care should be taken to avoid taking the life of the animals in the course of anesthesia. If one technician immobilizes the animal and extends the ear with tweezers while the other technician applies the solution, the procedure can be performed with accuracy without using anesthesia. If this approach is used six pairs of tweezers should be prepared, one for each group, to avoid contamination. Alternatively, the tweezers should be wiped with an alcohol swab after application is completed for each test group.

6.2 Days 2 and 3

Apply SLS solution and treatments using the same procedures as for Day 1.

When performing the application procedures the animals should be observed carefully for necrosis, hardening, hyperplasia or erythema of the auricula, as well as piloerection, or a decrease in locomotor activity. Any such abnormalities observed should be recorded on the test forms.

6.3 Day 7

On Day 7 the same procedures should be performed as on Days 1, 2, and 3.

Excision of the auricular lymph nodes will be performed from 24-30 hours after the start of application on Day 7. It is therefore recommended that application procedures on Day 7 begin in the morning or early afternoon.

7.0 Procedure on Test Day 8 (Excision of Auricular Lymph Nodes and ATP Assay)

7.1 Laboratory Preparation

Forty-eight 15 mL test tubes should each be filled with 1.98 mL of phosphate buffered saline (PBS). The dispensing of PBS should be conducted under aseptic manipulation. Dispense a minimum of 24 mL of PBS in a 50 mL test tube. Pipetting should be under aseptic manipulation.

Dissolve the luciferin-luciferase reagent according to the ATP assay kit instructions (at least 4.8 mL are required). The ATP assay kit provided, CheckLite™ 250 Plus,[4] includes five bottles each of luciferin-luciferase reagent, solvent water, and ATP releasing agent. Using one bottle of each type, create a solution according to the instructions (approximately 5.5 mL). Shield the assay solutions from light using aluminum foil and refrigerate until the time of use. Immediately before using, return to room temperature and remove the foil prior to use. Dispense 0.1 mL of the ATP releasing agent included in the ATP assay kit to each of the 48 luminometer tubes. ATP assay kit reagents should be dispensed using sterilized pipette tips under aseptic manipulation to avoid contamination with ATP and microorganisms.

7.2 Body Weight Measurement

Weigh the mice and record their body weights to the nearest 0.1 g on the test forms.

7.3 Auricular Lymph Node Excision and Weight Measurement

Perform procedures in **Sections 7.3**, **7.4** and **7.5** within 24 to 30 hours after the start of treatment application on Day 7. The necessary materials for procedures in **Sections 7.3**, **7.4** and **7.5** are given in **Annex Ia**.

Immediately after sacrificing the mice with ether anesthesia excise completely all auricular lymph nodes for each ear (there can be one or two auricular lymph nodes) as illustrated in **Figure C-I-1**. Place the excised lymph nodes for one animal in a disposable petri dish and immediately measure the wet weight to the nearest 0.1 mg with an analytical balance.

[4] For the intralaboratory validation study by Daicel Chemical Industries, Ltd. (Idehara et al. 2008; Idehara unpublished), only the ATP content for potassium dichromate was measured by the CheckLite™ 250 Plus Kit (Kikkoman Corporation, Japan) and the ViaLight® HS Kit (Lonza Rockland, Inc., USA) was used for determining the ATP content of all the other substances in the intralaboratory validation.

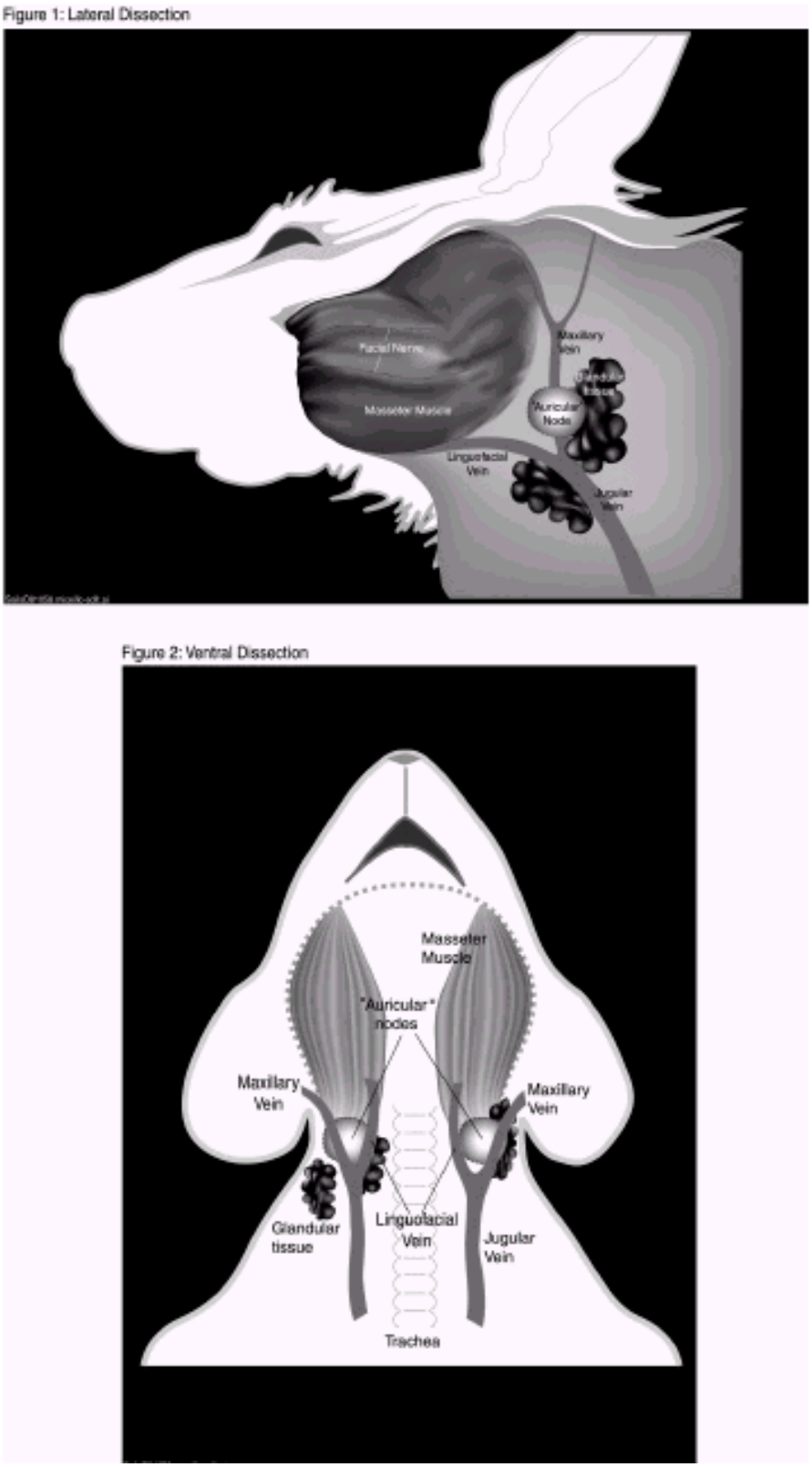
7.4 Preparation of Cell Suspension

The lymph nodes from one animal should be sandwiched between two pieces of slide glass and light pressure should be applied to crush the nodes (**Figure C-I-2**). After confirming that the tissue has spread out thinly pull the two slides apart. Suspend the tissue on both pieces of slide glass in 1 mL of PBS. As illustrated in **Figure C-I-3**, each piece of slide glass should be held at an angle over the petri dish and rinsed with PBS while the tissue is scraped off of the glass with repeated movements of a cell scraper. One mL of PBS should be used for rinsing both slides.

The tissue suspension in the petri dish should be homogenized lightly with the cell scraper, and 20 μL of the suspension should be taken up with a micropipette, taking care not to take up the membrane that is visible to the eye. The pipetted suspension should be added to 1.98 mL of PBS and homogenized well. This will be cell suspension No. 1. Again take up 20 μL of the suspension in the petri dish, add to 1.98 mL of PBS, and homogenize well. This will be cell suspension No. 2.

These procedures should be performed while wearing gloves and a mask, and micropipette tips should be sterile. Detailed step-by-step procedures are given in **Annex Ib**.

Figure C-I-1 Auricular lymph nodes[5]



[5] Taken from ICCVAM IWG LLNA Protocol (ICCVAM 2001).

Figure C-I-2 Preparation of cell suspension

Lymph nodes from each animal are sandwiched between two pieces of slide glass and light pressure is applied to crush the nodes.

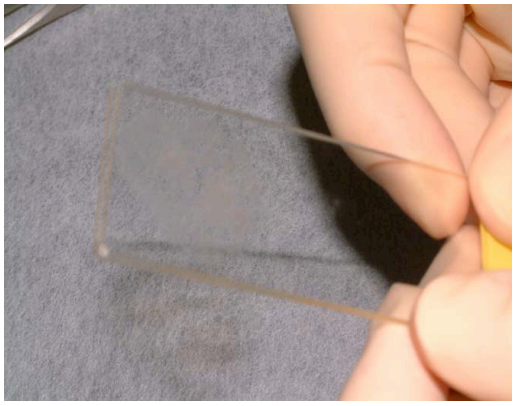
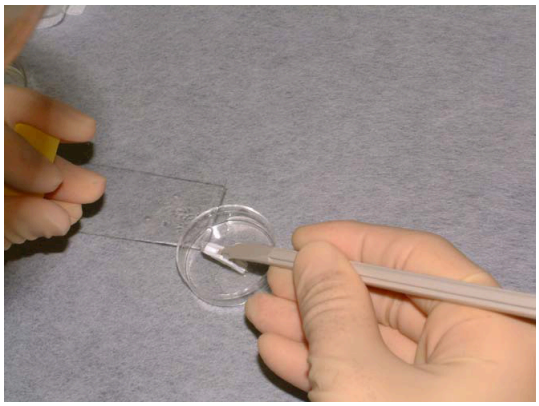


Figure C-I-3 Preparation of cell suspension

Rinse with PBS while scraping the tissue off of the glass with a cell scraper. Repeat the scraping motion, scooping up liquid from the petri dish as needed. Use 1 mL of PBS for the nodes of each animal.



7.5 ATP Assay

Prepare 48 luminometer tubes in advance by dispensing 0.1 mL of the ATP releasing reagent provided to each tube. Add 0.1 mL of each homogenized cell suspension to the luminometer tubes and homogenize. After allowing the solution in the tube to stand for approximately 20 seconds, add 0.1 mL of the luciferin-luciferase solution, promptly homogenize and place in the luminometer. The amount of bioluminescence (RLU; relative luminescence units) measured over 10 seconds will be displayed. Record this measurement on the test forms.

The amount of bioluminescence begins to decrease immediately after adding the luciferin-luciferase solution. It is therefore important that the series of procedures from the addition of luciferin-luciferase solution to switching on the luminometer are performed as quickly as possible, ideally with the same rhythm.

These procedures should be performed while wearing gloves and a mask, and micropipette tips should be sterile. The detailed procedures are given in **Annex Ic**.

8.0 Points of Caution on Procedures from Excision to ATP Assay

The ATP content of the lymph node decreases over time after the sacrifice of the animal. It is therefore desirable that the time elapsed between sacrifice of the animal and ATP assay is uniform for each animal. The series of procedures from excision to ATP assay must be performed rapidly and without delay.

If one technician performs these procedures, the animals should be sacrificed one at a time. If there are multiple technicians, it is possible to divide tasks and sacrifice the animals one group at a time. If two technicians perform the procedures, one individual should perform steps in **Section 7.3**, and the other individual should perform steps in **Sections 7.4** and **7.5**. If three technicians perform the procedures, one individual can handle steps in **Sections 7.3**, **7.4** and **7.5**. If multiple technicians are involved, it is important that the timing of excision is carefully planned so that there are no delays in subsequent steps.

9.0 Data Entry

Input the body weights on Day 1 and Day 8, the lymph node weight, and the amount of ATP bioluminescence into the designated Excel file.

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Annex Ia: Equipment and Reagents Used for the Experimental Procedures in Sections 7.3, 7.4, and 7.5

For the equipment and reagents underlined below, the items provided by the LLNA: DA Validation Committee should be used. In the event the test facility provides a luminometer, it can be used. Numbers in parentheses indicate the number of equipment or reagents required.

7.3 Auricular Lymph Node Excision and Weight Measurement

Dissecting instruments set (tweezers, scissors, surgical holder, injection needle and holder)

Antiseptic solution

Cotton

Petri dish (24)

Analytical balance (readability of at least 0.1 mg)

7.4 Preparation of Cell Suspension

15 mL test tubes with 1.98 mL PBS (48)

50 mL test tubes with at least 24 mL PBS (1)

Slide glass (48)

Tweezers (1)

Micropipette 1000 µL (1) (volume to be measured: 1 mL)

Micropipette 100 µL (1) (volume to be measured: 20 µL)

Cell scraper (1)

Sterilized pipette tips for 1000 µL micropipette (24) and for 100 µL micropipette (24)

Vortex mixer (1)

Paper towels

Clean sheet

Test tube rack

7.5 ATP Assay

Luminometer tubes with 0.1 mL ATP releasing agent (48)

15 mL test tube with dissolved luciferin-luciferase solution (1)

Micropipette – 100 µL or 200 µL (2) (volume to be measured: 0.1 mL)

Sterilized micropipette tips (96)

Timer (with second display) (1)

Luminometer (1)

Vortex mixer (can use same mixer listed under **Section 7.4** Preparation of Cell Suspension)

Test tube rack and luminometer tube rack (microtube rack)

Annex Ib: Preparation of Cell Suspension for the Experimental Procedures in Section 7.4

1. Cover the laboratory bench with a clean sheet and place one piece of slide glass on the sheet.
2. After measuring the lymph node weights, use tweezers to move the lymph nodes from one animal from the petri dish to the center of the slide glass.
3. Place another piece of slide glass on top.
4. Pick up the two sandwiched pieces of slide glass. Squeeze the two pieces in the center to crush the lymph nodes. (Apply only light pressure. Too much pressure can break the cells.)
5. Confirm that the tissue has spread out thinly between the two slides and place the sandwiched slides on the clean sheet.
6. Fasten a tip on the 1000 μL micropipette and draw 1 mL phosphate buffered saline (PBS) from the 50 mL tube.
7. Remove the upper slide glass from the sandwiched slides and place it on the clean sheet with the side that was in contact with the lymph node tissue facing up. The other slide glass should be held at an angle in the petri dish, the side with lymph node tissue affixed facing forward, and washed with 1 mL PBS.
8. Dispose of the 1000 μL micropipette tip.
9. Scrape the tissue off of the glass with a cell scraper, scooping up PBS from the petri dish and repeating the scraping motion. Confirm that there is no tissue, or only trace amounts of tissue, left on the slide before disposing of the slide glass.
10. Pick up the slide glass laid aside at step 7; scrape the tissue off in the same manner and dispose of the slide glass. Note that it becomes difficult to scrape the tissue off of the slide glass once it has dried. Perform steps 4-10 without delay. The scraping should be performed while keeping the area of the slide glass to which the lymph node tissue is affixed sufficiently wet with PBS from the petri dish.
11. The tissue suspension in the petri dish should be homogenized lightly with the cell scraper. If large pieces of tissue are observed, stir with the cell scraper to break up the pieces and obtain a uniform solution.
12. Wipe the cell scraper with a paper towel. (The cell scraper will be used for the next animal.)
13. Fasten a tip to the 100 μL micropipette, tilt the petri dish at an angle and mix the suspension by pipetting in and out several times. Take up 20 μL of the suspension with the pipette, taking care not to take up any membrane that is visible to the eye.
14. Add the 20 μL of suspension to a 15 mL test tube containing 1.98 mL PBS. Pipette the solution and proceed to homogenize with the vortex mixer. (cell suspension No. 1)
15. Repeat steps 13 and 14 to prepare cell suspension No. 2.
16. Dispose of the 100 μL micropipette tip.

Annex Ic: ATP Assay for the Experimental Procedures in Section 7.5

1. Fasten a tip on the 100 μL (or 200 μL) micropipette and draw 0.1 mL of vortex-homogenized cell suspension No. 1.
2. To the luminometer tube filled with 0.1 mL ATP releasing reagent, add 0.1 mL of cell suspension No. 1, making sure to note the time with a timer. Dispose of the tip.
3. Homogenize with the vortex mixer and place in the luminometer tube rack.
4. Fasten a tip on a separate 100 μL (or 200 μL) micropipette and draw 0.1 mL of solution from the 15 mL tube containing dissolved luciferin-luciferase reagent.
5. Take the luminometer tube from the rack and add 0.1 mL of luciferin-luciferase solution to the luminometer tube 20 seconds after the time noted in step 2.
6. Promptly homogenize in the vortex mixer, place in the luminometer and turn on the switch. The amount of bioluminescence begins to decrease immediately after adding the luciferin-luciferase solution. Step 6 should be performed as quickly as possible, ideally with the same rhythm.
7. Dispose of the tip.
8. After 10 seconds the amount of bioluminescence (RLU) will be displayed. Record this measurement on the test forms.
9. Repeat steps 1-8 for cell suspension No. 2, measure the bioluminescence and record.

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Annex I-2

LLNA: DA Test Method Data Comparing With and Without 1% SLS Pretreatment

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Table C-I-2-1 Summary of LLNA: DA Test Method Results Comparing With and Without 1% SLS Pretreatment[1]

Substance Name	Vehicle	Concentration (%)	SI[2] (+ SLS)	SI[2] (- SLS)	Calculated EC3[3] (+ SLS)	Calculated EC3[3] (- SLS)
2, 4-Dinitrochloro-benzene	AOO	0.03	2.10	1.88	0.05%	0.06%
		0.10	5.02	4.46		
		0.30	9.74	14.61		
Potassium dichromate	DMSO	0.1	2.61	2.54	0.15%	0.22%
		0.3	4.24	3.34		
		1.0	5.51	5.66		
Isoeugenol	AOO	1.0	2.05	1.32	2.46%	4.24%
		2.5	3.02	2.21		
		5.0	2.85	3.35		
Citral	AOO	5	1.93	1.88	7.4%	10.4%
		10	4.15	2.91		
		25	6.97	5.90		
Hexyl cinnamic aldehyde	AOO	5	1.51	0.99	7.5%	8.8%
		10	4.52	3.64		
		25	4.84	3.79		
Cinnamic alcohol	AOO	10	2.46	2.44	14.1%	18.5%
		25	4.40	3.43		
		50	6.36	4.01		
Hydroxycitronellal	AOO	10	1.98	1.49	15.8%	19.8%
		25	4.61	3.81		
		50	6.59	6.74		
Imidazolidinyl urea	DMF	10	2.36	2.54	20.3%	33.0%
		25	3.29	2.38		
		50	6.02	4.31		

Substance Name	Vehicle	Concentration (%)	SI[2] (+ SLS)	SI[2] (- SLS)	Calculated EC3[3] (+ SLS)	Calculated EC3[3] (- SLS)
Methyl methacrylate	AOO	25	0.73	1.11	NA	NA
		50	0.68	0.92		
		100	1.31	1.83		
Nickel (II) chloride	DMSO	2.5	1.53	0.98	NA	NA
		5.0	1.57	1.16		
		10.0	2.24	1.87		
Methyl salicylate	AOO	5	0.89	0.83	NA	NA
		10	1.59	1.32		
		25	1.69	2.34		
Salicylic acid	AOO	5	1.21	1.13	NA	NA
		10	2.05	1.29		
		25	2.48	2.44		
Sulfanilamide	DMF	10	1.08	0.92	NA	NA
		25	1.03	0.90		
		50	0.94	0.84		

Abbreviations: AOO = acetone: olive oil (4:1); DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; EC3 = estimated concentration required to produce a stimulation index of three; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; NA = not applicable; SI = stimulation index; SLS = sodium lauryl sulfate; + SLS = with pretreatment of 1% aqueous solution of SLS prior to test substance application; - SLS = without pretreatment of 1% aqueous solution of SLS prior to test substance application.

[1] Data submitted to NICEATM in February 2009 (Idehara unpublished).

[2] SI determined from mean ATP content (relative luminescence units).

[3] EC3 value was calculated based on interpolation or extrapolation formulas discussed in Gerberick et al. 2004.

Annex II

Physicochemical Properties and Chemical Classes of Substances Tested in the LLNA: DA

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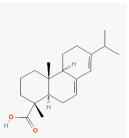
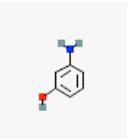
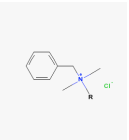
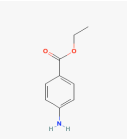
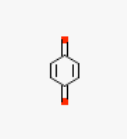
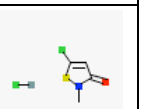
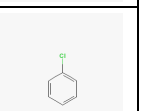
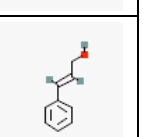
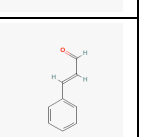
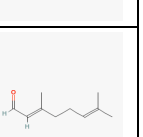
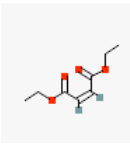
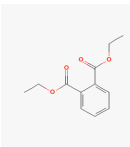
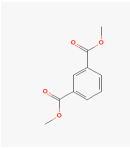
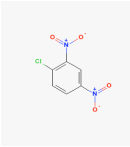
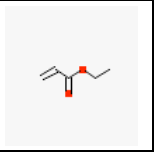
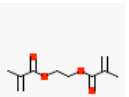
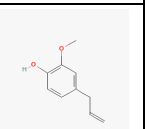
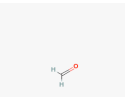
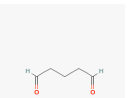
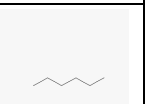
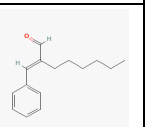
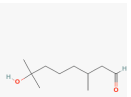
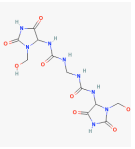
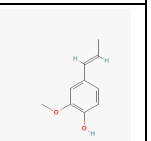
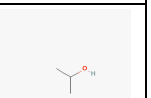
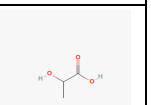
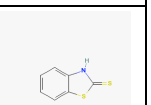
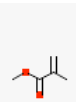
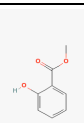
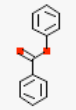
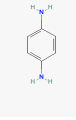
Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Abietic acid[5, 6]	Sylvic acid	514-10-3	302.46	11	NA	Solid	Hydrocarbons, cyclic; Polycyclic compounds	
3-Aminophenol[6]	*m*-Aminophenol	591-27-5	109.13	0.24	Minimal	Solid	Amines; Phenols	
Benzalkonium chloride[5]	Alkylbenzyldimethyl-ammonium chloride; Germitol; Zephiral	8001-54-5	170.66	NA	NA	Solid/ Liquid	Amines; Onium compounds	
Benzocaine[5]	Ethyl 4-aminobenzoate	94-09-7	165.19	1.80	NA	Solid	Carboxylic acids	
Benzoquinone[7]	*p*-Quinone; 1,4-benzoquinone; Cyclohexadienedione	106-51-4	108.10	1.17	High	Solid	Quinones	
1-Bromobutane[5]	Butyl bromide	109-65-9	137.02	2.65	Low	Liquid	Hydrocarbons, halogenated	

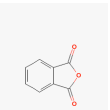
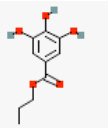
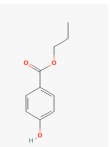
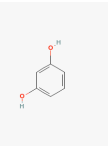
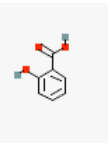
Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Butyl glycidyl ether[7]	*n*-Butyl glycidyl ether	2426-08-6	130.19	1.42	NA	Liquid	Ethers	
5-Chloro-2-methyl-4-isothiazolin-3-one[7]	Chloromethyliso-thiazolinone; CMI	26172-55-4	132.30	0.92	High	Liquid	Sulfur compounds; Heterocyclic compounds	
Chlorobenzene[5]	Phenyl chloride	108-90-7	112.56	2.64	Minimal	Liquid	Hydrocarbons, cyclic; Hydrocarbons, halogenated	
Cinnamic alcohol[7]	3-Phenyl-2-propen-1-ol; Cinnamyl alcohol	104-54-1	134.18	2.29	NA	Solid	Alcohols	
Cinnamic aldehyde[5]	Cinnamaldehyde	104-55-2	132.16	1.82	High	Liquid	Aldehydes	
Citral[5]	2,6-Octadienal, 3,7-dimethyl-	5392-40-5	152.24	3.45	High	Liquid	Hydrocarbons, other	

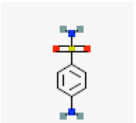
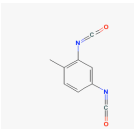
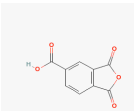
Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Cobalt chloride[5, 6, 8]	Cobaltous chloride	7646-79-9	129.84	0.85	NA	Solid	Inorganic chemical, elements; Inorganic chemical, metals	
Diethyl maleate[7]	Ethyl maleate	141-05-9	172.18	0.89	High	Liquid	Carboxylic acids	
Diethyl phthalate[5]	Ethyl phthalate; Phthalic acid, diethyl ester	84-66-2	222.24	2.65	Minimal	Liquid	Carboxylic acids	
Dimethyl isophthalate[6, 7]	1,3-Benzenedicarboxylic acid, dimethyl ester	1459-93-4	194.19	1.66	NA	Solid	Carboxylic acids	
2,4-Dinitrochloro-benzene[5, 6]	Dinitrochlorobenzene; DNCB	97-00-7	202.55	2.27	High	Solid	Hydrocarbons, cyclic; Hydrocarbons, halogenated; Nitro compounds	
Ethyl acrylate[7]	2-Propenoic acid, ethyl ester	140-88-5	100.10	NA	High	Liquid	Carboxylic acids	

Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Ethylene glycol dimethacrylate[7]	EGDMA	97-90-5	198.22	1.38	High	Liquid	Carboxylic acids	
Eugenol[5]	2-Methoxy-4-(2-propenyl)phenol; Allylguaiacol	97-53-0	164.20	2.73	NA	Liquid	Carboxylic acids	
Formaldehyde[5, 6]	Formalin	50-00-0	30.03	0.35	Moderate	Liquid	Aldehydes	
Glutaraldehyde[5, 6]	Glutaral; Pentanedial	111-30-8	100.12	-0.18	High	Liquid	Aldehydes	
Hexane[5]	Hexyl hydride; *n*-Hexane	110-54-3	86.18	3.29	Minimal	Liquid	Hydrocarbons, acyclic	
Hexyl cinnamic aldehyde[5, 6, 8]	alpha-Hexylcinnamaldehyde; HCA	101-86-0	216.32	4.82	Minimal	Liquid	Aldehydes	

Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Hydroxycitronellal[5]	Citronellal hydrate	107-75-5	172.26	2.11	Low	Liquid	Hydrocarbons, other	
Imidazolidinyl urea[5]	Germall 115; Imidurea	39236-46-9	388.30	-8.28	Moderate	Solid	Urea	
Isoeugenol[5, 6]	2-Methoxy-4-propenylphenol; 4-Propenylguaiacol	97-54-1	164.20	2.65	NA	Liquid	Carboxylic acids	
Isopropanol[5, 6]	Isopropyl alcohol; 2-Propanol	67-63-0	60.10	0.28	Minimal	Liquid	Alcohols	
Lactic acid[5, 8]	2-Hydroxypropanoic acid	50-21-5	90.08	-0.65	Minimal	Liquid	Carboxylic acids	
2-Mercaptobenzo-thiazole[5]	Captax	149-30-4	167.26	2.86	High	Solid	Heterocyclic compounds	

Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Methyl methacrylate[7]	MMA	80-62-6	100.12	NA	NA	Liquid	Carboxylic acids	
Methyl salicylate[5, 6]	Oil of wintergreen; Methyl 2-hydroxybenzoate	119-36-8	152.15	2.60	Minimal	Liquid	Carboxylic acids; Phenols	
Nickel (II) chloride[7]	Nickel chloride	7718-54-9	129.60	NA	NA	Solid	Inorganic chemical, elements; Inorganic chemical, metals	
Nickel (II) sulfate hexahydrate[5, 6, 8]	Nickel sulfate hexahydrate	10101-97-0	154.76	NA	NA	Solid	Inorganic chemical, elements; Inorganic chemical, metals	
Phenyl benzoate[7]	Diphenylcarboxylate	93-99-2	198.22	2.89	NA	Solid	Carboxylic acids	
p-Phenylenediamine[5]	4-Phenylenediamine	106-50-3	108.14	-0.39	NA	Solid	Amines	

Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Phthalic anhydride[5]	1,2-Benzenedicarboxylic anhydride; 1,3-Dioxophthalan	85-44-9	148.12	2.07	Moderate	Solid	Anhydrides; Carboxylic acids	
Potassium dichromate[5, 8]	PDC; Dipotassium bichromate	7778-50-9	294.18	-3.59	NA	Solid	Inorganic chemical, chromium compounds; Inorganic chemical, potassium compounds	
Propyl gallate[7]	Benzoic acid, 3,4,5-trihydroxy-, propyl ester; Gallic acid, propyl ester; Propyl 3,4,5-trihydroxybenzoate	121-79-9	212.20	NA	High	Solid	Carboxylic acids	
Propylparaben[5]	4-Hydroxybenzoic acid, propyl ester; Propyl p-hydroxybenzoate	94-13-3	180.20	2.98	Minimal	Solid	Carboxylic acids; Phenols	
Resorcinol[5]	1,3-Dihydroxybenzene	108-46-3	110.11	1.03	Minimal	Solid	Phenols	
Salicylic acid[7]	2-Hydroxybenzoic acid	69-72-7	138.12	1.03	NA	Solid	Phenols; Carboxylic acids	

Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Sodium lauryl sulfate[5]	Sodium dodecyl sulfate; SLS; SDS; Irium	151-21-3	288.38	1.69	NA	Solid	Alcohols; Sulfur compounds; Lipids	
Sulfanilamide[7]	4-Aminobenzene-sulfonamide; *p*-Anilinesulfonamide; *p*-Sulfamidoaniline	63-74-1	172.21	0.40	Minimal	Solid	Hydrocarbons, cyclic; Sulfur compounds	
Toluene 2,4-diisocyanate[5]	2,4-TDI	584-54-9	174.16	3.74	NA	Liquid	Hydrocarbons, cyclic; Isocyanates	
Trimellitic anhydride[5]	4-Carboxyphthalic anhydride	552-30-7	192.13	1.95	Low	Solid	Anhydrides; Carboxylic acids	

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; K_{ow} = octanol-water partition coefficient; Mol. = molecular; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; NA = not available.

[1] Total of 46 substances: intralaboratory validation study (Idehara et al. 2008; Idehara unpublished) tested 45 substances and the two-phased interlaboratory validation study (Omori et al. 2008) tested 14 substances (i.e., 13 of the 45 substances from the intralaboratory validation study and one unique substance not tested in the intralaboratory validation study).

[2] K_{ow} represents the estimated octanol-water partition coefficient (expressed on log scale) calculated by the Syracuse Research Corporation from the website: http://www.srcinc.com/what-we-do/databaseforms.aspx?id=385.

[3] Peptide reactivity based on Cys (1:10) and Lys (1:50) data as reported in Gerberick et al. 2004 and/or Gerberick et al. 2007.

[4] Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, as developed by the National Library of Medicine: http://www.nlm.nih.gov/mesh/meshhome.html.

[5] Substance tested in intralaboratory validation study (Idehara et al. 2008).

[6] Substance tested in phase one of two-phased interlaboratory validation study (Omori et al. 2008).

[7] Substance tested in intralaboratory validation study (Idehara unpublished).

[8] Substance tested in phase two of two-phased interlaboratory validation study (Omori et al. 2008).

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Annex III

Comparative LLNA: DA, Traditional LLNA, Guinea Pig, and Human Skin Sensitization Data

Annex III-1
Comparison of LLNA: DA, Traditional LLNA, Guinea Pig, and Human Results (Alphanumeric Order) C-123

Annex III-2
Comparison of Alternative LLNA: DA Decision Criteria and Traditional LLNA Results (Alphanumeric Order) C-141

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Annex III-1

Comparison of LLNA: DA, Traditional LLNA, Guinea Pig, and Human Results (Alphanumeric Order)

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Table C-III-1-1 Comparative Performance of the LLNA: DA, Traditional LLNA, Guinea Pig, and Human Tests (Alphanumeric Order)

Substance Name	Veh.[1]	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[2]	LLNA: DA Reference	Trad. LLNA Result[3]	GP Result[4]	Human Result[5]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritation Data	Skin Irritation Reference
Abietic acid	AOO	25	6.26	Idehara et al. 2008	+ (5.2, 25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 25% (GP)	Basketter et al. 2007b
Abietic acid	AOO	25	4.64	Omori et al. 2008	+ (5.2, 25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 25% (GP)	Basketter et al. 2007b
Abietic acid	AOO	25	7.96	Omori et al. 2008	+ (5.2, 25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 25% (GP)	Basketter et al. 2007b
Abietic acid	AOO	25	3.98 at 10%	Omori et al. 2008	+ (5.2, 25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 25% (GP)	Basketter et al. 2007b
3-Aminophenol	AOO	10	2.83	Omori et al. 2008	+ (5.7, 10%)	NA (+ nonstd)	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter et al. 2007b
3-Aminophenol	AOO	10	1.76 at 3%	Omori et al. 2008	+ (5.7, 10%)	NA (+ nonstd)	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter et al. 2007b
3-Aminophenol	AOO	10	2.38	Omori et al. 2008	+ (5.7, 10%)	NA (+ nonstd)	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter et al. 2007b
Benzalkonium chloride	AOO[6]	2.5	6.68	Idehara et al. 2008	+ (11.1, 2%)[7]	-	+	Gerberick 1992	ICCVAM 1999	ICCVAM 1999	Irritant at 2% in ACE (mice); irritant at 1% in ACE (mice)	Gerberick et al 2002; Manetz and Meade 1999
Benzocaine	**AOO**	**25**	**4.84**	**Idehara et al. 2008**	**+/- (7.6, 20%)[8]**	+	+/-	**ICCVAM 1999**	**ICCVAM 1999**	**Poole et al. 1970; ICCVAM 1999 (Equivocal data)**	**Negative at ≤ 10% (GP)**	**Basketter and Scholes 1992**

Substance Name	Veh.[1]	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[2]	LLNA: DA Reference	Trad. LLNA Result[3]	GP Result[4]	Human Result[5]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritation Data	Skin Irritation Reference
p-Benzo-quinone	AOO	0.100	3.79	Idehara unpublished	+ (52.3, 2.5%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at 2.5% (GP)	Basketter et al. 2007b
1-Bromo-butane	AOO	25	1.65	Idehara et al. 2008	- (1.2, 25%)[9]	NA	NA	ICCVAM 1999	NA	NA	NA	NA
Butyl glycidyl ether	AOO	50	4.59	Idehara unpublished	+ (5.6, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.1% (GP)	Wahlberg and Boman 1985
Chlorobenzene	AOO	25	2.44	Idehara et al. 2008	- (1.7, 25%)[9]	-	NA	ICCVAM 1999	ICCVAM 1999	NA	No data. Low irritancy potential assumed based on clinical literature.	Basketter et al. 1998
5-Chloro-2-methyl-4-isothiazolin-3-one	DMF	0.100	7.50	Idehara unpublished	+ (27.7, 0.1%)	+	+	ICCVAM 1999; Gerberick et al. 2005	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
Cinnamic alcohol	AOO	90	5.66 at 50%	Idehara unpublished	+ (5.7, 90%)	+	+	Gerberick et al. 2005	Robinson et al. 1990	Jordan and King 1977	Nonirritant at 1% (GP)	Robinson et al. 1990
Cinnamic aldehyde	AOO	15	4.73	Idehara et al. 2008	+ (18.4, 25%)[9]	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.75% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Citral	AOO	25	4.40	Idehara et al. 2008	+ (20.5, 20%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.5% (GP)	Basketter et al. 2007b
Cobalt chloride	DMSO	5	3.64	Idehara et al. 2008	+ (7.2, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes 1992

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Cobalt chloride	DMSO	1[10]	2.66	Omori et al. 2008	+ (7.2, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes 1992
Cobalt chloride	DMSO	3	20.55	Omori et al. 2008	+ (7.2, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes 1992
Cobalt chloride	DMSO	3	8.07	Omori et al. 2008	+ (7.2, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes 1992
Cobalt chloride	DMSO	5	2.01	Omori et al. 2008	+ (7.2, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes 1992
Cobalt chloride	DMSO	5	2.54	Omori et al. 2008	+ (7.2, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes 1992
Cobalt chloride	DMSO	5	4.25	Omori et al. 2008	+ (7.2, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes 1992
Cobalt chloride	DMSO	5	5.06	Omori et al. 2008	+ (7.2, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes 1992
Diethyl maleate	AOO	10.0	3.78	Idehara unpublished	+ (22.6, 100%)[11]	NA	+	Gerberick et al. 2005	NA	Marzulli and Maibach 1980	Nonirritant at 100% (GP)	Basketter et al. 2007b
Diethyl phthalate	AOO	100	1.09	Idehara et al. 2008	- (1.5, 100%)	-	+	Gerberick et al. 2005	Klecak et al. 1977	ICCVAM 1999	Negative at 100% (rabbits)	ECETOC 1995
Dimethyl isophthalate	AOO	25	0.89 at 5%	Idehara unpublished	- (1.0, 25%)	-	-	ICCVAM 1999; Basketter et al. 1999b	ICCVAM 1999; Basketter et al. 1999b	Basketter et al. 1999b	Negative at ≤ 10% (GP)	Basketter and Scholes 1992

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Dimethyl isophthalate	AOO	25	1.34 at 5%	Omori et al. 2008	- (1.0, 25%)	-	-	ICCVAM 1999; Basketter et al. 1999b	ICCVAM 1999; Basketter et al. 1999b	Basketter et al. 1999b	Negative at ≤ 10% (GP)	Basketter and Scholes 1992
Dimethyl isophthalate	AOO	25	1.00 at 5%	Omori et al. 2008	- (1.0, 25%)	-	-	ICCVAM 1999; Basketter et al. 1999b	ICCVAM 1999; Basketter et al. 1999b	Basketter et al. 1999b	Negative at ≤ 10% (GP)	Basketter and Scholes 1992
Dimethyl isophthalate	AOO	25	1.26 at 5%	Omori et al. 2008	- (1.0, 25%)	-	-	ICCVAM 1999; Basketter et al. 1999b	ICCVAM 1999; Basketter et al. 1999b	Basketter et al. 1999b	Negative at ≤ 10% (GP)	Basketter and Scholes 1992
2,4-Dinitrochloro-benzene	AOO	1	7.10	Idehara et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	AOO	0.30	11.97	Omori et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	AOO	0.30	9.23	Omori et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	AOO	0.30	9.96	Omori et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	AOO	0.30	8.53	Omori et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b

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2,4-Dinitrochloro-benzene	AOO	0.30	7.86	Omori et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	AOO	0.30	15.14	Omori et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	AOO	0.30	13.18	Omori et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	AOO	0.30	12.60	Omori et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	AOO	0.30	10.89	Omori et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	AOO	0.30	4.71	Omori et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
Ethyl acrylate	AOO	50	4.29 at 25%	Idehara unpublished	+ (4, 50%)	-	+	Gerberick et al. 2005	Van der Walle et al. 1982	Marzulli and Maibach 1974	Nonirritant at 0.3 M (GP)	Van der Walle et al. 1982
Ethylene glycol dimethacrylate	MEK	50	4.45	Idehara unpublished	+ (7, 50%)	-	+	ICCVAM 1999	ICCVAM 1999; Gerberick et al.1992	ICCVAM 1999; Basketter et al. 1999b	Nonirritant at 1% (GP)	Wahlberg and Boman 1985
Eugenol	AOO	25	7.07	Idehara et al. 2008	+ (17, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 25% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995

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Formaldehyde	ACE	2.50	5.10	Idehara et al. 2008	+ (4, 1.85%)	+	+	Gerberick et al. 2005; Hilton et al. 1998	ICCVAM 1999	ICCVAM 1999; Kwon et al. 2003	Nonirritant at 2% (GP)	Basketter et al. 2007b
Formaldehyde	ACE	5.0	4.84	Omori et al. 2008	+ (4, 1.85%)	+	+	Gerberick et al. 2005; Hilton et al. 1998	ICCVAM 1999	ICCVAM 1999; Kwon et al. 2003	Nonirritant at 2% (GP)	Basketter et al. 2007b
Formaldehyde	ACE	5.0	3.18	Omori et al. 2008	+ (4, 1.85%)	+	+	Gerberick et al. 2005; Hilton et al. 1998	ICCVAM 1999	ICCVAM 1999; Kwon et al. 2003	Nonirritant at 2% (GP)	Basketter et al. 2007b
Formaldehyde	ACE	5.0	2.69	Omori et al. 2008	+ (4, 1.85%)	+	+	Gerberick et al. 2005; Hilton et al. 1998	ICCVAM 1999	ICCVAM 1999; Kwon et al. 2003	Nonirritant at 2% (GP)	Basketter et al. 2007b
Glutaraldehyde	ACE	0.25	6.45	Idehara et al. 2008	+ (18, 2.5%)	+	+	Basketter et al. 2005; Hilton et al. 1998	Gad et al. 1986	Marzulli and Maibach 1974; Schneider and Akkan 2004	NA	NA
Glutaraldehyde	ACE	0.50	5.00	Omori et al. 2008	+ (18, 2.5%)	+	+	Basketter et al. 2005; Hilton et al. 1998	Gad et al. 1986	Marzulli and Maibach 1974; Schneider and Akkan 2004	NA	NA
Glutaraldehyde	ACE	0.50	3.39	Omori et al. 2008	+ (18, 2.5%)	+	+	Basketter et al. 2005; Hilton et al. 1998	Gad et al. 1986	Marzulli and Maibach 1974; Schneider and Akkan 2004	NA	NA
Glutaraldehyde	ACE	0.50	2.57	Omori et al. 2008	+ (18, 2.5%)	+	+	Basketter et al. 2005; Hilton et al. 1998	Gad et al. 1986	Marzulli and Maibach 1974; Schneider and Akkan 2004	NA	NA

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Hexane	AOO	100	2.31	Idehara et al. 2008	- (2.2, 100%)	NA	-	ICCVAM 1999	NA	ICCVAM 1999	Irritant at 100% (humans)	Kligman 1966c
Hexyl cinnamic aldehyde	AOO	25	6.47	Idehara et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	5.78	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	4.82	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	4.44	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	5.11	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	3.97	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	5.50	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	7.09	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995

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Hexyl cinnamic aldehyde	AOO	25	10.22	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	3.88	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	3.51	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	4.47	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	5.71	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	5.41	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	7.60	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	3.92	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	8.42	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995

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Hexyl cinnamic aldehyde	AOO	25	6.45	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hydroxy-citronellal	AOO	50	5.69	Idehara et al. 2008	+ (8.5, 100%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 50% (GP); negative at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Imidazolidinyl urea	DMF	50	4.67	Idehara et al. 2008	+ (5.5, 50%)	+	+	Gerberick et al. 2005	ICCVAM 1999	ICCVAM 1999	Negative at ≤75% (GP)	Basketter and Scholes 1992
Isoeugenol	AOO	50	12.36 at 25%	Idehara et al. 2008	+ (31, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter et al. 2007b
Isoeugenol	AOO	10	6.11	Omori et al. 2008	+ (31, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter et al. 2007b
Isoeugenol	AOO	10	5.54	Omori et al. 2008	+ (31, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter et al. 2007b
Isoeugenol	AOO	10	7.09	Omori et al. 2008	+ (31, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter et al. 2007b
Isopropanol	AOO	50	1.08 at 25%	Idehara et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	1.54 at 10%	Omori et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	0.91 at 10%	Omori et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	1.01 at 10%	Omori et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995

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Isopropanol	AOO	50	1.57 at 10%	Omori et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	0.76 at 25%	Omori et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	1.97 at 10%	Omori et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	1.45 at 10%	Omori et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	1.21 at 10%	Omori et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	0.70 at 25%	Omori et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	1.25	Omori et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995
Lactic acid	DMSO	50	1.06 at 10%	Idehara et al. 2008	- (2.2, 25%)	-	-	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Slightly irritating at 10% aq. (rabbits)	Cosmetic Ingredient Review Expert Panel 1998
Lactic acid	DMSO	25	0.93 at 5%	Omori et al. 2008	- (2.2, 25%)	-	-	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Slightly irritating at 10% aq. (rabbits)	Cosmetic Ingredient Review Expert Panel 1998
Lactic acid	DMSO	25	0.99 at 5%	Omori et al. 2008	- (2.2, 25%)	-	-	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Slightly irritating at 10% aq. (rabbits)	Cosmetic Ingredient Review Expert Panel 1998

Substance Name	Veh.[1]	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[2]	LLNA: DA Reference	Trad. LLNA Result[3]	GP Result[4]	Human Result[5]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritation Data	Skin Irritation Reference
Lactic acid	DMSO	25	0.97 at 10%	Omori et al. 2008	- (2.2, 25%)	-	-	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Slightly irritating at 10% aq. (rabbits)	Cosmetic Ingredient Review Expert Panel 1998
Lactic acid	DMSO	25	0.91	Omori et al. 2008	- (2.2, 25%)	-	-	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Slightly irritating at 10% aq. (rabbits)	Cosmetic Ingredient Review Expert Panel 1998
2-Mercaptobenzo-thiazole	DMF	50	2.00	Idehara et al. 2008	+ (8.6, 10%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 10% (GP); nonirritant at 25% (humans)	Basketter et al. 2007b; Kligman 1966c
Methyl methacrylate	AOO	100	1.81	Idehara unpublished	+ (3.6, 100%)	+	+ (case studies, no exposure concen-tration)	Betts et al. 2006	Van der Walle et al. 1982	Betts et al. 2006	Nonirritant at 3 M (GP)	Van der Walle et al. 1982
Methyl salicylate	AOO	25	1.20	Idehara et al. 2008	- (2.9, 20%)	-	-	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Irritant at 10% AOO (mice)	Gerberick et al 2002
Methyl salicylate	AOO	25	1.55	Omori et al. 2008	- (2.9, 20%)	-	-	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Irritant at 10% AOO (mice)	Gerberick et al 2002
Methyl salicylate	AOO	25	1.77 at 10%	Omori et al. 2008	- (2.9, 20%)	-	-	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Irritant at 10% AOO (mice)	Gerberick et al 2002
Methyl salicylate	AOO	25	0.83	Omori et al. 2008	- (2.9, 20%)	-	-	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Irritant at 10% AOO (mice)	Gerberick et al 2002
Nickel (II) chloride	DMSO	10	1.30	Idehara unpublished	- (2.4, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	Vandenberg and Epstein 1963	Negative at ≤ 0.15% (GP)	Basketter and Scholes 1992

Substance Name	Veh.[1]	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[2]	LLNA: DA Reference	Trad. LLNA Result[3]	GP Result[4]	Human Result[5]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritation Data	Skin Irritation Reference
Nickel (II) sulfate hexahydrate	DMSO	5.0	2.17 at 2.5%	Idehara et al. 2008	+ (3.1, 5%)	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Nickel (II) sulfate hexahydrate	DMSO	10	1.52 at 3%	Omori et al. 2008	+ (3.1, 5%)	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Nickel (II) sulfate hexahydrate	DMSO	10	11.78	Omori et al. 2008	+ (3.1, 5%)	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Nickel (II) sulfate hexahydrate	DMSO	10	3.49 at 1%	Omori et al. 2008	+ (3.1, 5%)	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Nickel (II) sulfate hexahydrate	DMSO	10	0.79 at 3%	Omori et al. 2008	+ (3.1, 5%)	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Nickel (II) sulfate hexahydrate	DMSO	10	1.24 at 3%	Omori et al. 2008	+ (3.1, 5%)	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Nickel (II) sulfate hexahydrate	DMSO	10	2.13	Omori et al. 2008	+ (3.1, 5%)	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Nickel (II) sulfate hexahydrate	DMSO	10	1.56 at 3%	Omori et al. 2008	+ (3.1, 5%)	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Phenyl benzoate	AOO	10	4.24 at 5%	Idehara unpublished	+ (11.1, 25%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al 2005	NA	NA

Substance Name	Veh.[1]	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[2]	LLNA: DA Reference	Trad. LLNA Result[3]	GP Result[4]	Human Result[5]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritation Data	Skin Irritation Reference
p-Phenylenediamine	AOO	1	5.14 at 0.25%	Idehara et al. 2008	+ (26, 1%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.5% (GP)	Basketter et al. 2007b
Phthalic anhydride	AOO	1.0	6.85	Idehara et al. 2008	+ (26.0, 10%)[12]	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 2001	Negative at ≤ 10% (GP)	Basketter and Scholes 1992
Potassium dichromate	DMSO	1.0	5.49	Idehara et al. 2008	+ (33.6, 0.5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.15% (GP)	Basketter et al. 2007b
Potassium dichromate	DMSO	1.0	4.78	Omori et al. 2008	+ (33.6, 0.5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.15% (GP)	Basketter et al. 2007b
Potassium dichromate	DMSO	1.0	4.08	Omori et al. 2008	+ (33.6, 0.5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.15% (GP)	Basketter et al. 2007b
Potassium dichromate	DMSO	1.0	6.01	Omori et al. 2008	+ (33.6, 0.5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.15% (GP)	Basketter et al. 2007b
Potassium dichromate	DMSO	1.0	6.37	Omori et al. 2008	+ (33.6, 0.5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.15% (GP)	Basketter et al. 2007b
Propyl gallate	AOO	2.5	4.95	Idehara unpublished	+ (33.6, 25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter and Scholes 1992
Propylparaben	AOO	25	1.28	Idehara et al. 2008	- (1.4, 25%)[13]	-	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 10% (GP)	Basketter and Scholes 1992
Resorcinol	AOO	25	4.33	Idehara et al. 2008	+ (10.4, 50%)	-	+	Basketter et al. 2007a	ICCVAM 1999	ICCVAM 1999; Basketter et al. 2007a	Nonirritant at 15% (humans)	Kligman 1966c
Salicylic acid	AOO	25	2.00	Idehara unpublished	- (2.5, 25%)	-	-	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Irritant at 20% aq. (mice)	Gerberick et al. 2002

Substance Name	Veh.[1]	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[2]	LLNA: DA Reference	Trad. LLNA Result[3]	GP Result[4]	Human Result[5]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritation Data	Skin Irritation Reference
Sodium lauryl sulfate	DMF	10	3.39	Idehara et al. 2008	+ (8.9, 20%)[9]	-	-	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Irritant at 20% (humans); Irritant at 20% (rabbits); irritant at 10% in DMF (mice)	Kligman 1966c; ECETOC 1995; Antonopoulos et al. 2008
Sulfanilamide	DMF	50	0.86 at 25%	Idehara unpublished	- (1, 50%)[14]	-	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Kligman 1966c	Nonirritant at 25% (humans)	Kligman 1966c
Toluene 2,4-diisocyanate	**AOO**	**0.25**	**9.43**	**Idehara et al. 2008**	**+[15] (NA)**	+	+	**van Och et al. 2001**	**NA**	**Basketter et al. 2001**	**NA**	**NA**
Trimellitic anhydride	AOO	0.50	4.96	Idehara et al. 2008	+ (4.6, 25%)	+	NA	ICCVAM 1999; Basketter and Scholes 1992	ICCVAM 1999; Gad et al. 1986	ICCVAM 1999; Basketter et al. 2001	Negative at ≤ 10% (GP)	Basketter and Scholes 1992

Bold Substances not included in accuracy analyses.

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); aq. = aqueous; CASRN = Chemical Abstracts Service Registry Number; Conc. = concentration; DMF = *N,N*-dimethylformamide; DMSO = dimethylsulfoxide; ECETOC = European Centre for Ecotoxicology and Toxicology of Chemicals; GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; MEK = methyl ethyl ketone; NA = not available; nonstd = nonstandard; SI = stimulation index; Trad. = traditional; "+" = Sensitizer.

"-" = Nonsensitizer.

[1] Applies to both traditional LLNA and LLNA: DA, unless otherwise noted.

[2] Highest SI occurred at highest concentration tested, unless otherwise noted.

[3] Numbers in parentheses indicate the highest SI and the highest concentration tested. Highest SI occurred at highest concentration tested, unless otherwise footnoted.

[4] GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.

[5] Human refers to outcomes obtained by studies conducted using the human maximization test, inclusion of the test substance in a human patch test allergen kit, and/or published clinical case studies/reports.

[6] Vehicle for traditional LLNA was acetone.

[7] Highest SI occurred at 1%.

[8] Equivocal traditional LLNA data (ICCVAM 1999); substance not included in accuracy analyses.

[9] Highest SI occurred at 10%.

[10] Data not reported for the highest dose (i.e., 3%), only for 0.3% and 1%.

[11] Highest SI occurred at 50%.

[12] Highest SI occurred at 2.5%.

[13] Highest SI occurred at 5%.

[14] Highest SI occurred at both 10% and 25%.

[15] Comparable LLNA reference data from modified LLNA test (van Och et al. 2000).

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Annex III-2

Comparison of Alternative LLNA: DA Decision Criteria and Traditional LLNA Results (Alphanumeric Order)

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Table C-III-2-1 Comparative Performance of Various LLNA: DA SI Values and Traditional LLNA Tests (Alphanumeric Order)

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	Stats.[2]	≥95 % CI	≥3 SD	≥2 SD	SI ≥											LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
								5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.8	1.5	1.3	1.0			
Abietic acid	514-10-3	25	6.26	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Abietic acid	514-10-3	25	4.64	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Abietic acid	514-10-3	25	7.96	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Abietic acid	514-10-3	25	3.98 at 10%	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
3-Amino-phenol	591-27-5	10	2.83	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
3-Amino-phenol	591-27-5	10	1.76 at 3%	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	Omori et al. 2008	+	ICCVAM 1999
3-Amino-phenol	591-27-5	10	2.38	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Benzalkonium chloride	8001-54-5	2.5	6.68	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	Gerberick et al. 1992
Benzocaine	**94-09-7**	**25**	**4.84**	**+**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**Idehara et al. 2008**	**+/-[3]**	**ICCVAM 1999**
p-Benzo-quinone	106-51-4	0.100	3.79	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Idehara unpublished	+	ICCVAM 1999
1-Bromo-butane	109-65-9	25	1.65	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	Idehara et al. 2008	-	ICCVAM 1999
Butyl glycidyl ether	2426-08-6	50	4.59	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	Idehara unpublished	+	ICCVAM 1999
Chlorobenzene	108-90-7	25	2.44	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	Idehara et al. 2008	-	ICCVAM 1999
5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	0.100	7.50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara unpublished	+	ICCVAM 1999; Gerberick et al. 2005

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	Stats.[2]	≥95 % CI	≥3 SD	≥2 SD	SI ≥											LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
								5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.8	1.5	1.3	1.0			
Cinnamic alcohol	104-54-1	90	5.66 at 50%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara unpublished	+	Gerberick et al. 2005
Cinnamic aldehyde	104-55-2	15	4.73	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Citral	5392-40-5	25	4.40	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	5	3.64	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	1[4]	2.66	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	3	20.55	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	3	8.07	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	5	2.01	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	5	2.54	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	5	4.25	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	5	5.06	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Diethyl maleate	141-05-9	10.0	3.78	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Idehara unpublished	+	Gerberick et al. 2005
Diethyl phthalate	84-66-2	100	1.09	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	Idehara et al. 2008	-	Gerberick et al. 2005
Dimethyl isophthalate	1459-93-4	25	0.89 at 5%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Idehara unpublished	-	ICCVAM 1999; Basketter et al. 1999b

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	Stats.[2]	≥95 % CI	≥3 SD	≥2 SD	SI ≥											LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
								5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.8	1.5	1.3	1.0			
Dimethyl isophthalate	1459-93-4	25	1.34 at 5%	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	Omori et al. 2008	-	ICCVAM 1999; Basketter et al. 1999b
Dimethyl isophthalate	1459-93-4	25	1.00 at 5%	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	Omori et al. 2008	-	ICCVAM 1999; Basketter et al. 1999b
Dimethyl isophthalate	1459-93-4	25	1.26 at 5%	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	Omori et al. 2008	-	ICCVAM 1999; Basketter et al. 1999b
2,4-Dinitrochloro-benzene	97-00-7	1	7.10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	11.97	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	9.23	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	9.96	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	8.53	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	7.86	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	15.14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	13.18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	12.60	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	10.89	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	Stats.[2]	≥95 % CI	≥3 SD	≥2 SD	SI ≥											LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
								5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.8	1.5	1.3	1.0			
2,4-Dinitrochloro-benzene	97-00-7	0.30	4.71	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Ethyl acrylate	140-88-5	50	4.29 at 25%	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	Idehara unpublished	+	Gerberick et al. 2005
Ethylene glycol dimethacrylate	97-90-5	50	4.45	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	Idehara unpublished	+	ICCVAM 1999
Eugenol	97-53-0	25	7.07	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Formaldehyde	50-00-0	2.50	5.10	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	Idehara et al. 2008	+	Gerberick et al. 2005; Hilton et al. 1998
Formaldehyde	50-00-0	5.0	4.84	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	Gerberick et al. 2005; Hilton et al. 1998
Formaldehyde	50-00-0	5.0	3.18	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	Omori et al. 2008	+	Gerberick et al. 2005; Hilton et al. 1998
Formaldehyde	50-00-0	5.0	2.69	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	Omori et al. 2008	+	Gerberick et al. 2005; Hilton et al. 1998
Glutaraldehyde	111-30-8	0.25	6.45	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	Basketter et al. 2005; Hilton et al. 1998
Glutaraldehyde	111-30-8	0.50	5.00	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	Basketter et al. 2005; Hilton et al. 1998
Glutaraldehyde	111-30-8	0.50	3.39	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	Omori et al. 2008	+	Basketter et al. 2005; Hilton et al. 1998
Glutaraldehyde	111-30-8	0.50	2.57	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	Omori et al. 2008	+	Basketter et al. 2005; Hilton et al. 1998

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	Stats.[2]	≥95 % CI	≥3 SD	≥2 SD	SI ≥										LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
								5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.8	1.5	1.3	1.0		
Hexane	110-54-3	100	2.31	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	Idehara et al. 2008	-	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	6.47	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	5.78	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	4.82	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	4.44	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	5.11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	3.97	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	5.50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	7.09	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	10.22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	3.88	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	3.51	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	4.47	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	5.71	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	5.41	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	Stats.[2]	≥95 % CI	≥3 SD	≥2 SD	SI ≥											LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
								5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.8	1.5	1.3	1.0			
Hexyl cinnamic aldehyde	101-86-0	25	7.60	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	3.92	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	8.42	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	6.45	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hydroxycitronellal	107-75-5	50	5.69	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Imidazolidinyl urea	39236-46-9	50	4.67	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	Gerberick et al. 2005
Isoeugenol	97-54-1	50	12.36 at 25%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Isoeugenol	97-54-1	10	6.11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Isoeugenol	97-54-1	10	5.54	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Isoeugenol	97-54-1	10	7.09	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Isopropanol	67-63-0	50	1.08 at 25%	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	Idehara et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.54 at 10%	-	+	-	+	-	-	-	-	-	-	-	-	+	+	+	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	0.91 at 10%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.01 at 10%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.57 at 10%	+	+	-	+	-	-	-	-	-	-	-	-	+	+	+	Omori et al. 2008	-	ICCVAM 1999

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	Stats.[2]	≥95 % CI	≥3 SD	≥2 SD	SI ≥											LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
								5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.8	1.5	1.3	1.0			
Isopropanol	67-63-0	50	0.76 at 25%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.97 at 10%	-	+	+	+	-	-	-	-	-	-	-	+	+	+	+	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.45 at 10%	-	+	-	+	-	-	-	-	-	-	-	-	-	+	+	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.21 at 10%	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	0.70 at 25%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.25	-	+	-	+	-	-	-	-	-	-	-	-	-	-	+	Omori et al. 2008	-	ICCVAM 1999
Lactic acid	50-21-5	50	1.06 at 10%	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	Idehara et al. 2008	-	ICCVAM 1999
Lactic acid	50-21-5	25	0.93 at 5%	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
Lactic acid	50-21-5	25	0.99 at 5%	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
Lactic acid	50-21-5	25	0.97 at 10%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
Lactic acid	50-21-5	25	0.91	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
2-Mercapto-benzothiazole	149-30-4	50	2.00	-	+	+	+	-	-	-	-	-	-	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Methyl methacrylate	80-62-6	100	1.81	-	+	-	-	-	-	-	-	-	-	-	+	+	+	+	Idehara unpublished	+	Betts et al. 2006
Methyl salicylate	119-36-8	25	1.20	-	+	-	+	-	-	-	-	-	-	-	-	-	-	+	Idehara et al. 2008	-	ICCVAM 1999
Methyl salicylate	119-36-8	25	1.55	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+	Omori et al. 2008	-	ICCVAM 1999

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	Stats.[2]	≥95 % CI	≥3 SD	≥2 SD	SI ≥											LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
								5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.8	1.5	1.3	1.0			
Methyl salicylate	119-36-8	25	1.77 at 10%	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	Omori et al. 2008	-	ICCVAM 1999
Methyl salicylate	119-36-8	25	0.83	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
Nickel (II) chloride	7718-54-9	10	1.30	-	+	+	+	-	-	-	-	-	-	-	-	-	+	+	Idehara unpublished	-	ICCVAM 1999
Nickel (II) sulfate hexahydrate	10101-97-0	5.0	2.17 at 2.5%	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	Idehara et al. 2008	+	Ryan et al. 2002
Nickel (II) sulfate hexahydrate	10101-97-0	10	1.52 at 3%	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	Omori et al. 2008	+	Ryan et al. 2002
Nickel (II) sulfate hexahydrate	10101-97-0	10	11.78	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	Ryan et al. 2002
Nickel (II) sulfate hexahydrate	10101-97-0	10	3.49 at 1%	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	Omori et al. 2008	+	Ryan et al. 2002
Nickel (II) sulfate hexahydrate	10101-97-0	10	0.79 at 3%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	+	Ryan et al. 2002
Nickel (II) sulfate hexahydrate	10101-97-0	10	1.24 at 3%	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	Omori et al. 2008	+	Ryan et al. 2002
Nickel (II) sulfate hexahydrate	10101-97-0	10	2.13	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	Omori et al. 2008	+	Ryan et al. 2002
Nickel (II) sulfate hexahydrate	10101-97-0	10	1.56 at 3%	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	Omori et al. 2008	+	Ryan et al. 2002
Phenyl benzoate	93-99-2	10.0	4.24 at 5%	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Idehara unpublished	+	ICCVAM 1999
p-Phenylene-diamine	106-50-3	1	5.14 at 0.25%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Phthalic anhydride	85-44-9	1.0	6.85	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	Stats.[2]	≥95 % CI	≥3 SD	≥2 SD	SI ≥ 5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.8	1.5	1.3	1.0	LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
Potassium dichromate	7778-50-9	1.0	5.49	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Potassium dichromate	7778-50-9	1.0	4.78	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Potassium dichromate	7778-50-9	1.0	4.08	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Potassium dichromate	7778-50-9	1.0	6.01	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Potassium dichromate	7778-50-9	1.0	6.37	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Propyl gallate	121-79-9	2.5	4.95	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	Idehara unpublished	+	ICCVAM 1999
Propylparaben	94-13-3	25	1.28	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	Idehara et al. 2008	-	ICCVAM 1999
Resorcinol	108-46-3	25	4.33	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	Basketter et al. 2007a
Salicylic acid	69-72-7	25	2.00	+	+	-	+	-	-	-	-	-	-	+	+	+	+	+	Idehara unpublished	-	ICCVAM 1999
Sodium lauryl sulfate	151-21-3	10	3.39	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Sulfanilamide	63-74-1	50	0.86 at 25%	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Idehara unpublished	-	ICCVAM 1999
Toluene 2,4-diisocyanate	**584-84-9**	**0.25**	**9.43**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	**Idehara et al. 2008**	+[5]	**Van Och et al. 2001**
Trimellitic anhydride	552-30-7	0.50	4.96	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999; Basketter and Scholes 1992

Entries in boldface indicate substances not included in accuracy analyses.

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; CI = confidence interval (mean ATP measurement of any treatment group is greater than 95% CI of mean ATP measurement for vehicle control group); Conc. = concentration; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP

content; Ref. = reference; SD = standard deviation (mean ATP measurement of any treatment group is greater than two or three SD for vehicle control group); SI = stimulation index; stats. = statistics (analysis of variance for multiple dose groups or *t*-test to compare one treatment group to the vehicle control group); Trad. = traditional.

"+" = Sensitizer.

"-" = Nonsensitizer.

[1] Highest SI occurred at highest concentration tested, unless otherwise noted.

[2] The ATP data were log-transformed prior to statistical analyses. For analysis of variance, significance at $p < 0.05$ was further tested by Dunnett's test.

[3] Equivocal (i.e., results that were not reproducible) traditional LLNA data (ICCVAM 1999). Substance not included in accuracy analyses.

[4] Data not reported for the highest dose (i.e., 3%), only for 0.3% and 1%.

[5] LLNA reference data from modified LLNA test (van Och et al. 2000). Substance not included in accuracy analyses.

Annex IV

Data for the LLNA: DA Intralaboratory and Interlaboratory Validation Studies

Annex IV-1
Individual Animal Data for the LLNA: DA (Intralaboratory)..... C-155

Annex IV-2
Summary Data for 14 Additional Substances Tested in the LLNA: DA (Intralaboratory).. C-173

Annex IV-3
Individual Animal Data for the LLNA: DA (Interlaboratory)..... C-179

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Annex IV-1

Individual Animal Data for the LLNA: DA (Intralaboratory)

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Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
VC	AOO	0	1	4927	1.12				
			2	3547	0.80				
			3	4758	1.08				
			Mean	**4411**	**1.00**				
PC - Eugenol	AOO	10	1	17020	3.86				
			2	14029	3.18				
			3	12117	2.75				
			Mean	**14388**	**3.26**				
Citral	AOO	5	1	9191	2.08	**15.63**	**12.46**	**5.96**	**4.11**
			2	12120	2.75				
			3	4808	1.09				
			Mean	**8706**	**1.97**				
		10	1	9937	2.25				
			2	7447	1.69				
			3	10528	2.39				
			Mean	**9304**	**2.11**				
		15	1	12297	2.79				
			2	11863	2.69				
			3	14283	3.24				
			Mean	**12814**	**2.91**				
		25	1	18200	4.13				
			2	22609	5.13				
			3	17469	3.96				
			Mean	**19426**	**4.40**				
Cinnamic aldehyde	AOO	1	1	6780	1.54	**2.98**	**2.08**	**0.92**	**0.63**
			2	13271	3.01				
			3	7545	1.71				
			Mean	**9199**	**2.09**				
		2.5	1	13624	3.09				
			2	8924	2.02				
			3	12681	2.88				
			Mean	**11743**	**2.66**				
		5	1	21945	4.98				
			2	17313	3.93				
			3	19218	4.36				
			Mean	**19492**	**4.42**				
		15	1	20037	4.54				
			2	18085	4.10				
			3	24421	5.54				
			Mean	**20848**	**4.73**				
VC	AOO	0	1	3759	0.97				
			2	3995	1.03				
			3	3461	0.89				
			4	4269	1.10				
			Mean	**3871**	**1.00**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
PC - Eugenol	AOO	10	1	16624	4.30				
			2	23785	6.15				
			3	15667	4.05				
			4	18066	4.67				
			Mean	**18535**	**4.79**				
Eugenol	AOO	5	1	12594	3.25	**4.50**	**3.60**	**2.88**	**2.63**
			2	15216	3.93				
			3	9790	2.53				
			4	NT	NT				
			Mean	**12533**	**3.24**				
		10	1	16624	4.30				
			2	23785	6.15				
			3	15667	4.05				
			4	18066	4.67				
			Mean	**18535**	**4.79**				
		25	1	26107	6.75				
			2	26713	6.90				
			3	29297	7.57				
			4	NT	NT				
			Mean	**27372**	**7.07**				
Propylparaben	AOO	5	1	5058	1.31	**NA**	**NA**	**NA**	**NA**
			2	4773	1.23				
			3	3034	0.78				
			Mean	**4288**	**1.11**				
		10	1	5539	1.43				
			2	3919	1.01				
			3	3713	0.96				
			Mean	**4390**	**1.13**				
		25	1	6385	1.65				
			2	5813	1.50				
			3	2679	0.69				
			Mean	**4959**	**1.28**				
Hexyl cinnamic aldehyde	AOO	5	1	7375	1.91	**11.62**	**9.69**	**7.75**	**6.97**
			2	3858	1.00				
			3	3782	1.00				
			Mean	**5005**	**1.29**				
		10	1	9217	2.38				
			2	12654	3.27				
			3	8072	2.09				
			Mean	**9981**	**2.58**				
		25	1	30420	7.86				
			2	27682	7.15				
			3	17014	4.40				
			Mean	**25038**	**6.47**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
Methyl salicylate	AOO	5	1	3250	0.84	**NA**	**NA**	**NA**	**NA**
			2	3310	0.86				
			3	1760	0.46				
			Mean	**2773**	**0.72**				
		10	1	4499	1.16				
			2	4637	1.20				
			3	2035	0.53				
			Mean	**3723**	**0.96**				
		25	1	4542	1.17				
			2	5445	1.41				
			3	3996	1.03				
			Mean	**4661**	**1.20**				
VC 1	AOO	0	1	3529	1.17				
			2	3106	1.03				
			3	2949	0.98				
			4	2473	0.82				
			Mean	**3014**	**1.00**				
PC 1 - Eugenol	AOO	10	1	20105	6.67				
			2	14663	4.87				
			3	14233	4.72				
			4	13137	4.36				
			Mean	**15535**	**5.15**				
VC 2	DMSO	0	1	4770	0.72				
			2	6914	1.04				
			3	8487	1.27				
			4	6527	0.98				
			Mean	**6674**	**1.00**				
PC 2 - Eugenol	DMSO	10	1	10887	1.63				
			2	16454	2.47				
			3	9982	1.50				
			4	12245	1.84				
			Mean	**12392**	**1.86**				
Abietic acid	AOO	5	1	4143	1.38	**7.90**	**5.99**	**4.40**	**3.96**
			2	9059	3.01				
			3	7056	2.34				
			Mean	**6752**	**2.24**				
		10	1	13190	4.30				
			2	8354	2.77				
			3	10561	3.50				
			Mean	**10701**	**3.55**				
		25	1	20693	6.87				
			2	17109	5.68				
			3	18770	6.23				
			Mean	**18857**	**6.26**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
Cobalt II chloride	DMSO	1	1	17709	2.65	**3.27**	**1.94**	**0.88**	**0.70**
			2	12673	1.90				
			3	12428	1.86				
			Mean	**14270**	**2.14**				
		2.5	1	17680	2.65				
			2	17863	2.68				
			3	18809	2.82				
			Mean	**18117**	**2.71**				
		5	1	28248	4.23				
			2	27268	4.09				
			3	17378	2.60				
			Mean	**24298**	**3.64**				
Nickel (II) sulfate hexahydrate	DMSO	1	1	7672	1.15	**NA**	**NA**	**2.18**	**1.81**
			2	11041	1.65				
			3	8581	1.29				
			Mean	**9098**	**1.36**				
		2.5	1	10829	1.62				
			2	10925	1.64				
			3	21735	3.26				
			Mean	**14496**	**2.17**				
		5	1	15969	2.39				
			2	9433	1.41				
			3	11636	1.74				
			Mean	**12346**	**1.85**				
VC 1	AOO	0	1	2660	1.03				
			2	2856	1.11				
			3	1828	0.71				
			4	2975	1.15				
			Mean	**2580**	**1.00**				
PC 1 - Eugenol	AOO	10	1	19298	7.48				
			2	17360	6.73				
			3	14953	5.80				
			4	11827	4.59				
			Mean	**15859**	**6.15**				
VC 2	DMF	0	1	4424	1.29				
			2	3087	0.90				
			3	2348	0.69				
			4	3854	1.12				
			Mean	**3428**	**1.00**				
PC 2 - Eugenol	DMF	10	1	5738	1.67				
			2	5644	1.65				
			3	3688	1.08				
			4	8185	2.39				
			Mean	**5813**	**1.70**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
Benzocaine	AOO	5	1	10495	4.07	**6.57**	**4.66**	**3.49**	**3.11**
			2	3052	1.18				
			3	6751	2.62				
			Mean	**6766**	**2.62**				
		10	1	10314	4.00				
			2	10880	4.22				
			3	8378	3.25				
			Mean	**9857**	**3.82**				
		25	1	10512	4.08				
			2	14366	5.57				
			3	12564	4.87				
			Mean	**12480**	**4.84**				
Imidazolidinyl urea	DMF	10	1	7333	2.14	**18.77**	**11.94**	**7.42**	**6.28**
			2	6777	1.98				
			3	10143	2.96				
			Mean	**8084**	**2.36**				
		25	1	9854	2.88				
			2	13907	4.06				
			3	11783	3.44				
			Mean	**11848**	**3.46**				
		50	1	14760	4.31				
			2	15299	4.46				
			3	17971	5.24				
			Mean	**16010**	**4.67**				
2-Mercaptobenzothiazole	DMF	10	1	7829	2.28	**NA**	**NA**	**9.99**	**7.99**
			2	7102	2.07				
			3	5647	1.65				
			Mean	**6859**	**2.00**				
		25	1	6978	2.04				
			2	2425	0.71				
			3	4401	1.28				
			Mean	**4601**	**1.34**				
		50	1	3976	1.16				
			2	4375	1.28				
			3	2675	0.78				
			Mean	**3675**	**1.07**				
VC	AOO	0	1	1453	0.28				
			2	11748	2.27				
			3	4663	0.90				
			4	2810	0.54				
			Mean	**5168**	**1.00**				
PC - Eugenol	AOO	10	1	13351	2.58				
			2	27023	5.23				
			3	12875	2.49				
			4	15921	3.08				
			Mean	**17292**	**3.35**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
2-4-Dinitrochlorobenzene	AOO	0.03	1	11884	2.30	**0.16**	**0.13**	**0.11**	**0.08**
			2	11146	2.16				
			3	5799	1.12				
			Mean	**9610**	**1.86**				
		0.05	1	10848	2.10				
			2	7394	1.43				
			3	8468	1.64				
			Mean	**8903**	**1.72**				
		0.1	1	13205	2.56				
			2	8679	1.68				
			3	6740	1.30				
			Mean	**9541**	**1.85**				
		0.25	1	34300	6.64				
			2	26924	5.21				
			3	15631	3.03				
			Mean	**25618**	**4.96**				
		0.5	1	33092	6.40				
			2	46685	9.03				
			3	30241	5.85				
			Mean	**36673**	**7.10**				
		1	1	40795	7.89				
			2	36807	7.12				
			3	32445	6.29				
			Mean	**36682**	**7.10**				
VC	AOO	0	1	1460	0.41				
			2	5137	1.46				
			3	3988	1.13				
			Mean	**3528**	**1.00**				
PC - Eugenol	AOO	10	1	22813	6.47				
			2	21142	5.99				
			3	30985	8.78				
			Mean	**24980**	**7.08**				
Isoeugenol	AOO	2.5	1	15638	4.43	**2.35**	**1.79**	**1.36**	**1.22**
			2	9113	2.58				
			3	8197	2.32				
			Mean	**10982**	**3.11**				
		5	1	15773	4.47				
			2	19726	5.59				
			3	10920	3.10				
			Mean	**15473**	**4.39**				
		10	1	24776	7.02				
			2	23236	6.59				
			3	23595	6.69				
			Mean	**23869**	**6.77**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
Isoeugenol (continued)		25	1	40328	11.43				
			2	50432	14.30				
			3	40035	11.35				
			Mean	**43598**	**12.36**				
		50	1	43389	12.30				
			2	28424	8.06				
			3	40263	11.41				
			Mean	**37359**	**10.59**				
VC	AOO	0	1	836	0.55				
			2	1815	1.20				
			3	1752	1.16				
			4	1631	1.08				
			Mean	**1508**	**1.00**				
PC - Eugenol	AOO	10	1	13707	9.09				
			2	6746	4.47				
			3	10475	6.95				
			4	6855	4.54				
			Mean	**9446**	**6.26**				
Benzalkonium chloride	AOO	0.5	1	3027	2.01	**0.52**	**0.46**	**0.42**	**0.40**
			2	5780	3.83				
			3	4183	2.77				
			Mean	**4330**	**2.87**				
		1	1	9672	6.41				
			2	7809	5.18				
			3	10868	7.21				
			Mean	**9449**	**6.26**				
		2.5	1	10292	6.82				
			2	11879	7.88				
			3	8070	5.35				
			Mean	**10080**	**6.68**				
VC	DMF	0	1	2926	1.10				
			2	1674	0.63				
			3	3984	1.49				
			4	2091	0.78				
			Mean	**2668**	**1.00**				
PC - Cinnamic aldehyde	DMF	5	1	17595	6.59				
			2	12322	4.62				
			3	10331	3.87				
			4	12297	4.61				
			Mean	**13136**	**4.92**				
Sodium lauryl sulfate	DMF	1	1	3870	1.45	**6.88**	**2.91**	**1.91**	**1.64**
			2	2899	1.09				
			3	3777	1.42				
			Mean	**3515**	**1.32**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
Sodium lauryl sulfate (continued)		2.5	1	7965	2.99				
			2	4802	1.80				
			3	6838	2.56				
			Mean	**6535**	**2.45**				
		5	1	2945	1.10				
			2	7161	2.68				
			3	7913	2.97				
			Mean	**6006**	**2.25**				
		10	1	10337	3.87				
			2	6881	2.58				
			3	9932	3.72				
			Mean	**9050**	**3.39**				
VC	AOO	0	1	2045	0.97				
			2	1990	0.94				
			3	2212	1.05				
			4	2212	1.05				
			Mean	**2115**	**1.00**				
PC - Hexyl cinnamic aldehyde	AOO	15	1	14020	6.63				
			2	9078	4.29				
			3	8912	4.21				
			Mean	**10670**	**5.05**				
Isopropanol	AOO	10	1	1364	0.65	**NA**	**NA**	**NA**	**NA**
			2	2872	1.36				
			3	2417	1.14				
			Mean	**2218**	**1.05**				
		25	1	3820	1.81				
			2	1746	0.83				
			3	1298	0.61				
			Mean	**2288**	**1.08**				
		50	1	2249	1.06				
			2	700	0.33				
			3	2454	1.16				
			Mean	**1801**	**0.85**				
VC	AOO	0	1	2386	0.76				
			2	2967	0.95				
			3	4347	1.39				
			4	2816	0.90				
			Mean	**3129**	**1.00**				
PC - Hexyl cinnamic aldehyde	AOO	15	1	9352	2.99				
			2	16201	5.18				
			3	10538	3.37				
			4	9135	2.92				
			Mean	**11306**	**3.61**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
Hexane	AOO	25	1	3755	1.20	**NA**	**NA**	**89.19**	**82.22**
			2	3240	1.04				
			3	3136	1.00				
			Mean	**3377**	**1.08**				
		50	1	3070	0.98				
			2	2491	0.80				
			3	2658	0.85				
			Mean	**2740**	**0.88**				
		100	1	9027	2.89				
			2	6802	2.17				
			3	5850	1.87				
			Mean	**7226**	**2.31**				
VC	AOO	0	1	2370	0.84				
			2	3124	1.11				
			3	2314	0.82				
			4	3464	1.23				
			Mean	**2818**	**1.00**				
PC - Hexyl cinnamic aldehyde	AOO	15	1	7739	2.75				
			2	10867	3.86				
			3	5290	1.88				
			4	8570	3.04				
			Mean	**8116**	**2.88**				
Toluene-2,4-diisocyanate	AOO	0.05	1	9445	3.35	**0.05**	**0.04**	**0.04**	**0.03**
			2	11471	4.07				
			3	5999	2.13				
			Mean	**8972**	**3.18**				
		0.1	1	12732	4.52				
			2	17962	6.38				
			3	16204	5.75				
			Mean	**15632**	**5.55**				
		0.25	1	25104	8.91				
			2	27791	9.86				
			3	26785	9.51				
			Mean	**26560**	**9.43**				
VC	AOO	0	1	1727	0.80				
			2	2122	0.99				
			3	2111	0.98				
			4	2645	1.23				
			Mean	**2151**	**1.00**				
PC - Hexyl cinnamic aldehyde	AOO	15	1	14931	6.94				
			2	15575	7.24				
			3	13043	6.06				
			4	11199	5.21				
			Mean	**13687**	**6.36**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
1-Bromobutane	AOO	5	1	2701	1.26	**NA**	**NA**	**NA**	**NA**
			2	2491	1.16				
			3	4272	1.99				
			Mean	**3154**	**1.47**				
		10	1	1810	0.84				
			2	2130	0.99				
			3	878	0.41				
			Mean	**1606**	**0.75**				
		25	1	3483	1.62				
			2	2916	1.36				
			3	4220	1.96				
			Mean	**3539**	**1.65**				
Chlorobenzene	AOO	5	1	1875	0.87	**NA**	**NA**	**20.09**	**17.88**
			2	2180	1.01				
			3	1088	0.51				
			Mean	**1714**	**0.80**				
		10	1	2505	1.16				
			2	1840	0.86				
			3	2682	1.25				
			Mean	**2342**	**1.09**				
		25	1	2848	1.32				
			2	5302	2.47				
			3	7615	3.54				
			Mean	**5255**	**2.44**				
Diethyl phthalate	AOO	25	1	1543	0.72	**NA**	**NA**	**NA**	**NA**
			2	2561	1.19				
			3	2906	1.35				
			Mean	**2336**	**1.09**				
		50	1	1781	0.83				
			2	1371	0.64				
			3	2477	1.15				
			Mean	**1876**	**0.87**				
		100	1	1808	0.84				
			2	1288	0.60				
			3	2139	0.99				
			Mean	**1745**	**0.81**				
Hydroxycitronellal	AOO	10	1	5201	2.42	**13.74**	**11.21**	**9.23**	**8.67**
			2	4094	1.90				
			3	5293	2.46				
			Mean	**4862**	**2.26**				
		25	1	9519	4.43				
			2	13562	6.31				
			3	10656	4.95				
			Mean	**11246**	**5.23**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
Hydroxycitronellal (continued)		50	1	14400	6.70				
			2	8741	4.06				
			3	13563	6.31				
			Mean	**12234**	**5.69**				
VC	ACE	0	1	2232	1.39				
			2	1509	0.94				
			3	1287	0.80				
			4	1419	0.88				
			Mean	**1611**	**1.00**				
PC - Hexyl cinnamic aldehyde	ACE	15	1	13901	8.63				
			2	16265	10.09				
			3	15531	9.64				
			4	15749	9.77				
			Mean	**15361**	**9.53**				
Glutaraldehyde	ACE	0.05	1	1821	1.13	**0.10**	**0.09**	**0.07**	**0.07**
			2	2181	1.35				
			3	1931	1.12				
			Mean	**1978**	**1.23**				
		0.1	1	5389	3.34				
			2	2496	1.55				
			3	6344	3.94				
			Mean	**4743**	**2.94**				
		0.25	1	16484	10.20				
			2	6814	4.23				
			3	7889	4.90				
			Mean	**10396**	**6.45**				
VC	AOO	0	1	3101	0.92				
			2	3253	0.97				
			3	2687	0.80				
			4	4407	1.31				
			Mean	**3362**	**1.00**				
PC - Hexyl cinnamic aldehyde	AOO	15	1	22800	6.78				
			2	16696	4.97				
			3	17973	5.35				
			4	18757	5.58				
			Mean	**19056**	**5.67**				
Trimellitic anhydride	AOO	0.1	1	5681	1.69	**0.17**	**0.11**	**0.07**	**0.06**
			2	7841	2.33				
			3	11293	3.36				
			Mean	**8272**	**2.46**				
		0.25	1	13902	4.14				
			2	11270	3.35				
			3	10963	3.26				
			Mean	**12045**	**3.58**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
Trimellitic anhydride (continued)		0.5	1	14361	4.27				
			2	18976	5.64				
			3	16673	4.96				
			Mean	**16670**	**4.96**				
Phthalic anhydride	AOO	0.1	1	11304	3.36	**0.08**	**0.06**	**0.04**	**0.03**
			2	13066	3.89				
			3	12448	3.70				
			Mean	**12272**	**3.65**				
		0.25	1	8332	2.48				
			2	15717	4.68				
			3	9833	2.93				
			Mean	**11294**	**3.36**				
		0.5	1	22051	6.56				
			2	12828	3.82				
			3	24315	7.23				
			Mean	**19731**	**5.87**				
		1	1	19987	5.95				
			2	32118	9.55				
			3	17006	5.09				
			Mean	**23037**	**6.85**				
VC 1	DMSO	0	1	13832	1.36				
			2	9930	0.97				
			3	9958	0.98				
			4	7097	0.70				
			Mean	**10204**	**1.00**				
PC 1 - Hexyl cinnamic aldehyde	DMSO	15	1	17741	1.74				
			2	18810	1.84				
			3	18045	1.77				
			4	12293	1.21				
			Mean	**16722**	**1.64**				
Lactic acid	DMSO	5	1	6741	0.66	**NA**	**NA**	**NA**	**NA**
			2	12789	1.25				
			3	12217	1.12				
			Mean	**10582**	**1.04**				
		10	1	11054	1.08				
			2	11929	1.17				
			3	9542	0.94				
			Mean	**10841**	**1.06**				
		25	1	7025	0.69				
			2	13796	1.35				
			3	8677	0.85				
			Mean	**9832**	**0.96**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
Lactic acid (continued)		50	1	8623	0.85				
			2	10101	0.99				
			3	11594	1.14				
			Mean	**10106**	**0.99**				
VC 2	AOO	0	1	5263	1.07				
			2	4970	1.01				
			3	5431	1.11				
			4	3965	0.81				
			Mean	**4907**	**1.00**				
PC 2 - Hexyl cinnamic aldehyde	AOO	15	1	25796	5.26				
			2	24279	4.95				
			3	13979	2.85				
			4	23991	4.89				
			Mean	**22011**	**4.49**				
Resorcinol	AOO	5	1	12461	2.54	**6.44**	**5.09**	**4.20**	**3.90**
			2	11743	2.39				
			3	12095	2.47				
			Mean	**12099**	**2.47**				
		10	1	25798	5.26				
			2	16771	3.42				
			3	21121	4.30				
			Mean	**21230**	**4.33**				
		25	1	20760	4.23				
			2	21215	4.32				
			3	9659	1.97				
			Mean	**17211**	**3.51**				
VC	ACE	0	1	3937	1.45				
			2	2374	0.88				
			3	2360	0.87				
			4	2173	0.80				
			Mean	**2711**	**1.00**				
PC - Hexyl cinnamic aldhedye	ACE	15	1	21117	7.79				
			2	19843	7.32				
			3	12203	4.50				
			4	13734	5.07				
			Mean	**16724**	**6.17**				
Formaldehyde	ACE	0.1	1	5222	1.93	**1.16**	**0.81**	**0.44**	**0.29**
			2	3045	1.12				
			3	2923	1.08				
			Mean	**3730**	**1.38**				
		0.25	1	6167	2.28				
			2	2933	1.08				
			3	5093	1.88				
			Mean	**4731**	**1.75**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
Formaldehyde (continued)		0.5	1	2317	0.86				
			2	4479	1.65				
			3	5263	1.94				
			Mean	**4019**	**1.48**				
		1	1	7846	2.90				
			2	10628	3.92				
			3	3894	1.44				
			Mean	**7456**	**2.75**				
		2.5	1	17242	6.36				
			2	14355	5.30				
			3	9904	3.65				
			Mean	**13833**	**5.10**				
VC	DMSO	0	1	82453	1.27				
			2	78192	1.21				
			3	42838	0.66				
			4	56114	0.87				
			Mean	**64899**	**1.00**				
PC	NT	NT	1	NT	NT				
			2	NT	NT				
			3	NT	NT				
			4	NT	NT				
			Mean	**NT**	**NT**				
Potassium dichromate	DMSO	0.1	1	193231	2.98	**0.14**	**0.09**	**0.07**	**0.06**
			2	140171	2.16				
			3	186039	2.87				
			4	152378	2.35				
			Mean	**167954**	**2.59**				
		0.3	1	209189	3.22				
			2	274466	4.23				
			3	421230	6.49				
			Mean	253302	3.90				
		0.3	1	**289546**	**4.46**				
			2	286418	4.41				
			3	304081	4.69				
			Mean	440493	6.79				
		1	1	394755	6.08				
			2	**356437**	**5.49**				
VC	AOO	0	1	4172	1.44				
			2	3078	1.06				
			3	2136	0.74				
			4	2192	0.76				
			Mean	**2894**	**1.00**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
PC - Hexyl cinnamic aldehyde	AOO	15	1	10569	3.65				
			2	11027	3.81				
			3	12928	4.47				
			4	12520	4.33				
			Mean	**11761**	**4.06**				
p-Phenylenediamine	AOO	0.1	1	8259	2.85	**0.07**	**0.05**	**0.04**	**0.04**
			2	11194	3.87				
			3	11454	3.96				
			Mean	**10302**	**3.56**				
		0.25	1	12197	4.21				
			2	15785	5.45				
			3	16610	5.74				
			Mean	**14864**	**5.14**				
		0.5	1	16392	5.66				
			2	9781	3.38				
			3	10173	3.52				
			Mean	**12115**	**4.19**				
		1	1	10644	3.68				
			2	10669	3.69				
			3	5942	2.05				
			Mean	**9085**	**3.14**				

Abbreviations: ACE = acetone; Anim. = Animal; AOO = acetone: olive oil (4:1); ATP = adenosine triphosphate; Calc. = calculated; Conc. = concentration; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; EC3 = estimated concentration needed to produce a stimulation index of three; EC2.5 = estimated concentration needed to produce a stimulation index of 2.5; EC2 = estimated concentration needed to produce a stimulation index of two; EC1.8 = estimated concentration needed to produce a stimulation index of 1.8; NA = not applicable; No. = number; NT = not tested; PC = positive control; SI = stimulation index; VC = vehicle control; Veh. = vehicle.

[1] Original laboratory records with individual animal data for the 31 substances tested in the LLNA: DA intralaboratory validation study (Idehara et al. 2008) provided by Kenji Idehara, Ph.D., Daicel Chemical Industries, Ltd.

[2] The 31 substances in the intralaboratory validation study were evaluated during one of 18 LLNA: DA tests that were conducted between July 2003 through September 2007 and are listed in order based on the date that they were tested.

[3] Two ATP measurements were taken for each animal and the mean ATP is indicated.

[4] EC3 value was calculated based on interpolation or extrapolation formulas discussed in Gerberick et al. 2004.

[5] EC value (i.e., EC1.8, EC2, or EC2.5) was calculated based on modified interpolation or extrapolation formulas for EC3 values discussed in Gerberick et al. 2004.

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Annex IV-2

Summary Data for 14 Additional Substances Tested in the LLNA: DA (Intralaboratory)

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Table C-IV-2-1 Summary of the Results for 14 Additional Substances Tested in the LLNA: DA (Intralaboratory)[1]

Substance Name	Vehicle	Concentration (%)	SI[2]	Calculated EC3 (%)[3]	Calculated EC2.5 (%)[4]	Calculated EC2 (%)[4]	Calculated EC1.8 (%)[4]
5-Chloro-2-methyl-4-isothiazolin-3-one (CMI)	DMF	0.005	1.2	0.031	0.021	0.011	0.008
		0.010	1.9				
		0.025	2.7				
		0.050	4.0				
		0.100	7.5				
p-Benzoquinone	AOO	0.005	2.6	0.063	0.005	0.003	0.003
		0.010	2.6				
		0.025	2.5				
		0.050	2.7				
		0.100	3.8				
Propyl gallate	AOO	0.5	2.8	1.094	0.421	0.281	0.225
		1.0	2.9				
		2.5	4.9				
Phenyl benzoate	AOO	1.0	2.2	2.255	1.440	0.795	0.652
		2.5	3.2				
		5.0	4.2				
		10.0	3.7				
Diethyl maleate	AOO	0.5	1.9	3.705	2.084	1.181	0.889
		1.0	1.9				
		2.5	2.7				
		5.0	3.3				
		10.0	3.8				
Ethyl acrylate	AOO	10	2.5	13.943	9.793	7.537	6.788
		25	4.3				
		50	3.4				

Substance Name	Vehicle	Concentration (%)	SI[2]	Calculated EC3 (%)[3]	Calculated EC2.5 (%)[4]	Calculated EC2 (%)[4]	Calculated EC1.8 (%)[4]
Cinnamic alcohol	AOO	10	2.4	21.341	12.195	6.540	5.230
		25	3.2				
		50	5.7				
		90	4.4				
Ethylene glycol dimethacrylate	MEK	10	1.2	34.031	28.524	22.273	19.242
		25	2.2				
		50	4.4				
Butyl glycidyl ether	AOO	10	1.2	31.682	25.922	19.919	17.500
		25	2.4				
		50	4.6				
Nickel (II) chloride	DMSO	2.5	0.9	NA	NA	NA	NA
		5.0	1.1				
		10.0	1.3				
Salicylic acid	AOO	5	1.5	NA	NA	25.000	17.683
		10	1.6				
		25	2.0				
Sulfanilamide	DMF	10	0.8	NA	NA	NA	NA
		25	0.9				
		50	0.6				
Methyl methacrylate	AOO	25	1.0	NA	NA	NA	NA
		50	1.2				
		75	1.3				
		100	1.8				
Dimethyl isophthalate[5]	AOO	5	0.9	NA	NA	NA	NA
		10	0.9				
		25	0.8				

Abbreviations: AOO = acetone: olive oil (4:1); DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; EC3 = estimated concentration needed to produce a stimulation index of three; EC2.5 = estimated

concentration needed to produce a stimulation index of 2.5; EC2 = estimated concentration needed to produce a stimulation index of two; EC1.8 = estimated concentration needed to produce a stimulation index of 1.8; MEK = methyl ethyl ketone; NA = not applicable; SI = stimulation index.

[1] Original laboratory records with individual animal data for the 14 additional substances tested in the LLNA: DA intralaboratory validation study (Idehara unpublished) provided by Kenji Idehara, Ph.D., Daicel Chemical Industries, Ltd.

[2] SI determined from mean ATP content (relative luminescence units).

[3] EC3 value was calculated based on interpolation or extrapolation formulas discussed in Gerberick et al. 2004.

[4] EC value (i.e., EC2.5, EC2, or EC1.8) was calculated based on modified interpolation or extrapolation formulas for EC3 value discussed in Gerberick et al. 2004.

[5] This substance was also tested in the first phase of the interlaboratory validation study (Omori et al. 2008).

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Annex IV-3

Individual Animal Data for the LLNA: DA (Interlaboratory)

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Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
1	Vehicle - Positive Control		0	1	27373	1.09				
				2	23473	0.93				
				3	30778	1.22				
				4	19231	0.76				
				Mean	**25214**	**1.00**				
1	Positive Control		NA	1	163662	6.49				
				2	118724	4.71				
				3	120098	4.76				
				4	172911	6.86				
				Mean	**143849**	**5.71**				
1	Vehicle – Substance	AOO	0	1	30365	1.24				
				2	26124	1.06				
				3	25218	1.03				
				4	16624	0.68				
				Mean	**24583**	**1.00**				
1	Hexyl cinnamic aldehyde	AOO	5	1	39462	1.61	**9.98**	**8.47**	**6.96**	**6.36**
				2	29952	1.22				
				3	37759	1.54				
				4	25613	1.04				
				Mean	**33196**	**1.35**				
			10	1	94155	3.83				
				2	60720	2.47				
				3	70595	2.87				
				4	70068	2.85				
				Mean	**73884**	**3.01**				
			25	1	174255	7.09				
				2	140034	5.70				
				3	103168	4.20				
				4	151064	6.15				
				Mean	**142130**	**5.78**				
1	Isopropanol	AOO	10	1	49049	2.00	**NA**	**NA**	**NA**	**NA**
				2	46692	1.90				
				3	22501	0.92				
				4	32783	1.33				
				Mean	**37756**	**1.54**				
			25	1	28917	1.18				
				2	28183	1.15				
				3	28099	1.14				
				4	23206	0.94				
				Mean	**27101**	**1.10**				
			50	1	32979	1.34				
				2	28219	1.15				
				3	28788	1.17				
				4	24907	1.01				
				Mean	**28723**	**1.17**				
1	Vehicle - Positive Control		0	1	27603	1.19				
				2	29165	1.26				
				3	13867	0.60				
				4	21857	0.95				
				Mean	**23123**	**1.00**				
1	Positive Control		NA	1	187061	8.09				
				2	192723	8.33				
				3	152209	6.58				
				4	120141	5.20				
				Mean	**163033**	**7.05**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
1	Vehicle - Substance	ACE	0	1	23522	1.31				
				2	17328	0.97				
				3	19286	1.07				
				4	11653	0.65				
				Mean	**17947**	**1.00**				
1	Glutaraldehyde	ACE	0.05	1	39029	2.17	**0.11**	**0.09**	**0.07**	**0.06**
				2	21473	1.20				
				3	17442	0.97				
				4	24434	1.36				
				Mean	**25594**	**1.43**				
			0.15	1	86407	4.81				
				2	69645	3.88				
				3	44897	2.50				
				4	90044	5.02				
				Mean	**72748**	**4.05**				
			0.50	1	117767	6.56				
				2	91139	5.08				
				3	85284	4.75				
				4	64878	3.62				
				Mean	**89767**	**5.00**				
1	Formaldehyde	ACE	0.5	1	54229	3.02	**1.75**	**0.39**	**0.26**	**0.21**
				2	65863	3.67				
				3	49268	2.75				
				4	39499	2.20				
				Mean	**52214**	**2.91**				
			1.5	1	65799	3.67				
				2	35118	1.96				
				3	48274	2.69				
				4	56430	3.14				
				Mean	**51405**	**2.86**				
			5.0	1	92516	5.16				
				2	131184	7.31				
				3	52728	2.94				
				4	71309	3.97				
				Mean	**86934**	**4.84**				
1	Vehicle - Positive Control		0	1	25568	1.13				
				2	30989	1.37				
				3	15244	0.68				
				4	18525	0.82				
				Mean	**22582**	**1.00**				
1	Positive Control		NA	1	160326	7.10				
				2	97979	4.34				
				3	126572	5.61				
				4	151977	6.73				
				Mean	**134213**	**5.94**				
1	Vehicle - Substance	AOO	0	1	36866	1.36				
				2	33905	1.25				
				3	15218	0.56				
				4	22764	0.84				
				Mean	**27188**	**1.00**				
1	2,4-Dinitrochloro-benzene	AOO	0.03	1	108431	3.99	**0.03**	**0.03**	**0.02**	**0.02**
				2	83821	3.08				
				3	68037	2.50				
				4	48931	1.80				
				Mean	**77305**	**2.84**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
1	2,4-Dinitrochloro-benzene (continued)		0.10	1	185139	6.81				
				2	159188	5.86				
				3	133437	4.91				
				4	110880	4.08				
				Mean	**147161**	**5.41**				
			0.30	1	334363	12.30				
				2	258002	9.49				
				3	366438	13.48				
				4	343140	12.62				
				Mean	**325485**	**11.97**				
1	Dimethyl isophthalate	AOO	5	1	41322	1.52	**NA**	**NA**	**NA**	**NA**
				2	32753	1.20				
				3	24319	0.89				
				4	47742	1.76				
				Mean	**36534**	**1.34**				
			10	1	46499	1.71				
				2	27887	1.03				
				3	29565	1.09				
				4	20851	0.77				
				Mean	**31200**	**1.15**				
			25	1	39741	1.46				
				2	21245	0.78				
				3	38401	1.41				
				4	20734	0.76				
				Mean	**30030**	**1.10**				
1	3-Aminophenol	AOO	1	1	48998	1.80	**NA**	**5.49**	**1.88**	**1.17**
				2	50122	1.84				
				3	47237	1.74				
				4	44007	1.62				
				Mean	**47591**	**1.75**				
			3	1	65491	2.41				
				2	55831	2.05				
				3	55478	2.04				
				4	75285	2.77				
				Mean	**63021**	**2.32**				
			10	1	93723	3.45				
				2	57142	2.10				
				3	82054	3.02				
				4	74792	2.75				
				Mean	**76927**	**2.83**				
2	Vehicle - Positive Control		0	1	29854	0.94				
				2	36425	1.15				
				3	42387	1.34				
				4	18060	0.57				
				Mean	**31681**	**1.00**				
2	Positive Control		NA	1	194745	6.15				
				2	196510	6.20				
				3	202311	6.39				
				4	171703	5.42				
				Mean	**191317**	**6.04**				
2	Vehicle - Substance	AOO	0	1	26727	0.65				
				2	62370	1.51				
				3	48632	1.18				
				4	27029	0.66				
				Mean	**41189**	**1.00**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
2	Hexyl cinnamic aldehyde	AOO	5	1	49355	1.20	**12.41**	**9.41**	**7.46**	**6.69**
				2	57775	1.40				
				3	62556	1.52				
				4	55479	1.35				
				Mean	**56291**	**1.37**				
			10	1	129128	3.13				
				2	98419	2.39				
				3	96062	2.33				
				4	113209	2.75				
				Mean	**109204**	**2.65**				
			25	1	259210	6.29				
				2	185538	4.50				
				3	176096	4.28				
				4	173235	4.21				
				Mean	**198520**	**4.82**				
2	Isopropanol	AOO	10	1	48933	1.19	**NA**	**NA**	**NA**	**NA**
				2	26716	0.65				
				3	38147	0.93				
				4	35351	0.86				
				Mean	**37286**	**0.91**				
			25	1	40741	0.99				
				2	33529	0.81				
				3	36625	0.89				
				4	29201	0.71				
				Mean	**35024**	**0.85**				
			50	1	31132	0.76				
				2	44432	1.08				
				3	30372	0.74				
				4	27101	0.66				
				Mean	**33259**	**0.81**				
2	Vehicle - Positive Control		0	1	16450	0.51				
				2	56211	1.74				
				3	29690	0.92				
				4	26911	0.83				
				Mean	**32315**	**1.00**				
2	Positive Control		NA	1	100365	3.11				
				2	144864	4.48				
				3	121515	3.76				
				4	131149	4.06				
				Mean	**124473**	**3.85**				
2	Vehicle - Substance	AOO	0	1	26982	1.03				
				2	26503	1.01				
				3	23078	0.88				
				4	28074	1.07				
				Mean	**26159**	**1.00**				
2	2,4-Dinitrochloro-benzene	AOO	0.03	1	46482	1.78	**0.11**	**0.06**	**0.02**	**0.02**
				2	45109	1.72				
				3	64419	2.46				
				4	87361	3.34				
				Mean	**60843**	**2.33**				
			0.10	1	54947	2.10				
				2	79087	3.02				
				3	103400	3.95				
				4	44369	1.70				
				Mean	**70451**	**2.69**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
2	2,4-Dinitrochloro-benzene		0.30	1	154655	5.91				
				2	244903	9.36				
				3	231793	8.86				
				4	334511	12.79				
				Mean	**241465**	**9.23**				
2	Abietic acid	AOO	5	1	53429	2.04	**8.20**	**6.41**	**4.76**	**3.64**
				2	44953	1.72				
				3	55417	2.12				
				4	66359	2.54				
				Mean	**55039**	**2.10**				
			10	1	76437	2.92				
				2	106616	4.08				
				3	106351	4.07				
				4	77421	2.96				
				Mean	**91706**	**3.51**				
			25	1	109226	4.18				
				2	165358	6.32				
				3	78960	3.02				
				4	131863	5.04				
				Mean	**121351**	**4.64**				
2	Vehicle - Positive Control		0	1	15977	0.59				
				2	29941	1.11				
				3	25288	0.94				
				4	36217	1.35				
				Mean	**26856**	**1.00**				
2	Positive Control		NA	1	105933	3.94				
				2	170707	6.36				
				3	134656	5.01				
				4	173488	6.46				
				Mean	**146196**	**5.44**				
2	Vehicle - Substance	ACE	0	1	56525	1.49				
				2	38645	1.02				
				3	28667	0.75				
				4	28339	0.74				
				Mean	**38044**	**1.00**				
2	Glutaraldehyde	ACE	0.05	1	34115	0.90	**0.44**	**0.35**	**0.27**	**0.24**
				2	37388	0.98				
				3	17955	0.47				
				4	22926	0.60				
				Mean	**28096**	**0.74**				
			0.15	1	50405	1.32				
				2	36212	0.95				
				3	54707	1.44				
				4	54598	1.44				
				Mean	**48980**	**1.29**				
			0.50	1	172747	4.54				
				2	104608	2.75				
				3	105731	2.78				
				4	133355	3.51				
				Mean	**129110**	**3.39**				
2	Formaldehyde	ACE	0.5	1	71257	1.87	**1.48**	**1.11**	**0.73**	**0.58**
				2	61368	1.61				
				3	74954	1.97				
				4	50290	1.32				
				Mean	**64467**	**1.69**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
2	Formaldehyde (continued)		1.5	1	120557	3.17				
				2	110027	2.89				
				3	139716	3.67				
				4	90274	2.37				
				Mean	**115143**	**3.03**				
			5.0	1	148089	3.89				
				2	111959	2.94				
				3	97241	2.56				
				4	126577	3.33				
				Mean	**120966**	**3.18**				
3	Vehicle - Positive Control		0	1	14012	0.68				
				2	25742	1.25				
				3	18482	0.90				
				4	24206	1.17				
				Mean	**20610**	**1.00**				
3	Positive Control		NA	1	147051	7.13				
				2	129657	6.29				
				3	119376	5.79				
				4	132756	6.44				
				Mean	**132210**	**6.41**				
3	Vehicle - Substance	AOO	0	1	22801	0.95				
				2	28208	1.17				
				3	19180	0.80				
				4	26000	1.08				
				Mean	**24047**	**1.00**				
3	Methyl salicylate	AOO	5	1	22109	0.92	**NA**	**NA**	**NA**	**NA**
				2	22812	0.95				
				3	21410	0.89				
				4	36725	1.53				
				Mean	**25764**	**1.07**				
			10	1	35176	1.46				
				2	22115	0.92				
				3	21251	0.88				
				4	26904	1.12				
				Mean	**26361**	**1.10**				
			25	1	53142	2.21				
				2	31027	1.29				
				3	31120	1.29				
				4	34146	1.42				
				Mean	**37359**	**1.55**				
3	3-Aminophenol	AOO	1	1	40069	1.67	**NA**	**NA**	**NA**	**NA**
				2	31036	1.29				
				3	28933	1.20				
				4	35464	1.47				
				Mean	**33875**	**1.41**				
			3	1	51109	2.13				
				2	34706	1.44				
				3	53201	2.21				
				4	30394	1.26				
				Mean	**42352**	**1.76**				
			10	1	39746	1.65				
				2	38143	1.59				
				3	35330	1.47				
				4	53816	2.24				
				Mean	**41759**	**1.74**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
3	Vehicle - Positive Control		0	1	32037	1.14				
				2	27673	0.98				
				3	25512	0.91				
				4	27174	0.97				
				Mean	**28099**	**1.00**				
3	Positive Control		NA	1	133836	4.76				
				2	122152	4.35				
				3	164019	5.84				
				4	133810	4.76				
				Mean	**138454**	**4.93**				
3	Vehicle - Substance	AOO	0	1	52047	1.46				
				2	31377	0.88				
				3	36296	1.02				
				4	22887	0.64				
				Mean	**35652**	**1.00**				
3	Hexyl cinnamic aldehyde	AOO	5	1	38213	1.07	**14.90**	**11.39**	**8.40**	**7.35**
				2	35942	1.01				
				3	68561	1.92				
				4	50818	1.43				
				Mean	**48383**	**1.36**				
			10	1	69749	1.96				
				2	85956	2.41				
				3	97018	2.72				
				4	75438	2.12				
				Mean	**82040**	**2.30**				
			25	1	124915	3.50				
				2	168780	4.73				
				3	188378	5.28				
				4	151145	4.24				
				Mean	**158304**	**4.44**				
3	Isopropanol	AOO	10	1	32440	0.91	**NA**	**NA**	**NA**	**NA**
				2	45395	1.27				
				3	38482	1.08				
				4	28304	0.79				
				Mean	**36155**	**1.01**				
			25	1	30325	0.85				
				2	27645	0.78				
				3	23613	0.66				
				4	12277	0.34				
				Mean	**23465**	**0.66**				
			50	1	29038	0.81				
				2	28736	0.81				
				3	37489	1.05				
				4	28026	0.79				
				Mean	**30822**	**0.86**				
3	Vehicle - Positive Control		0	1	19428	0.70				
				2	34843	1.26				
				3	30475	1.11				
				4	25568	0.93				
				Mean	**27578**	**1.00**				
3	Positive Control		NA	1	152890	5.54				
				2	150397	5.45				
				3	179030	6.49				
				4	164124	5.95				
				Mean	**161610**	**5.86**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
3	Vehicle - Substance	AOO	0	1	27832	0.78				
				2	43858	1.23				
				3	39077	1.10				
				4	31673	0.89				
				Mean	**35610**	**1.00**				
3	2,4-Dinitrochloro-benzene	AOO	0.03	1	78157	2.19	**0.06**	**0.04**	**0.03**	**0.02**
				2	124013	3.48				
				3	79811	2.24				
				4	40213	1.13				
				Mean	**80548**	**2.26**				
			0.10	1	121518	3.41				
				2	178885	5.02				
				3	152199	4.27				
				4	149717	4.20				
				Mean	**150579**	**4.23**				
			0.30	1	333041	9.35				
				2	332166	9.33				
				3	364546	10.24				
				4	388959	10.92				
				Mean	**354678**	**9.96**				
3	Dimethyl isophthalate	AOO	5	1	31045	0.87	**NA**	**NA**	**NA**	**NA**
				2	35735	1.00				
				3	28933	0.81				
				4	47129	1.32				
				Mean	**35710**	**1.00**				
			10	1	42990	1.21				
				2	26663	0.75				
				3	27736	0.78				
				4	40039	1.12				
				Mean	**34357**	**0.96**				
			25	1	21801	0.61				
				2	20892	0.59				
				3	29220	0.82				
				4	23687	0.67				
				Mean	**23900**	**0.67**				
4	Vehicle - Positive Control		0	1	48083	1.06				
				2	39428	0.87				
				3	55411	1.22				
				4	38284	0.85				
				Mean	**45301**	**1.00**				
4	Positive Control		NA	1	211896	4.68				
				2	262733	5.80				
				3	242739	5.36				
				4	275773	6.09				
				Mean	**248285**	**5.48**				
4	Vehicle - Substance	DMSO	0	1	132462	1.32				
				2	79967	0.80				
				3	82192	0.82				
				4	106964	1.07				
				Mean	**100396**	**1.00**				
4	Cobalt chloride	DMSO	0.3	1	175468	1.75	**NA**	**0.82**	**0.28**	**0.23**
				2	192922	1.92				
				3	230415	2.30				
				4	216774	2.16				
				Mean	**203895**	**2.03**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
4	Cobalt chloride (continued)		1.0	1	272071	2.71				
				2	206730	2.06				
				3	333152	3.32				
				4	256734	2.56				
				Mean	**267172**	**2.66**				
			NA	1	NA	NA				
				2	NA	NA				
				3	NA	NA				
				4	NA	NA				
				Mean	**NA**	**NA**				
4	Nickel (II) sulfate hexahydrate	DMSO	1	1	136287	1.36	**NA**	**NA**	**NA**	**NA**
				2	84335	0.84				
				3	125617	1.25				
				4	118828	1.18				
				Mean	**116266**	**1.16**				
			3	1	152054	1.51				
				2	166405	1.66				
				3	188337	1.88				
				4	105499	1.05				
				Mean	**153074**	**1.52**				
			10	1	129555	1.29				
				2	89825	0.89				
				3	85180	0.85				
				4	109822	1.09				
				Mean	**103595**	**1.03**				
4	Vehicle - Positive Control		0	1	42028	0.90				
				2	49964	1.07				
				3	44351	0.95				
				4	50162	1.08				
				Mean	**46626**	**1.00**				
4	Positive Control		NA	1	266538	5.72				
				2	297022	6.37				
				3	208438	4.47				
				4	238300	5.11				
				Mean	**252574**	**5.42**				
4	Vehicle - Substance	AOO	0	1	38814	0.90				
				2	40081	0.93				
				3	36876	0.86				
				4	56256	1.31				
				Mean	**43007**	**1.00**				
4	Hexyl cinnamic aldehyde	AOO	5	1	66346	1.54	**9.34**	**7.90**	**6.46**	**5.8**
				2	63590	1.48				
				3	71486	1.66				
				4	55427	1.29				
				Mean	**64212**	**1.49**				
			10	1	92375	2.15				
				2	128592	2.99				
				3	121376	2.82				
				4	213148	4.96				
				Mean	**138873**	**3.23**				
			25	1	183245	4.26				
				2	237260	5.52				
				3	208440	4.85				
				4	249803	5.81				
				Mean	**219687**	**5.11**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
4	Isopropanol	AOO	10	1	62566	1.45	**NA**	**NA**	**NA**	**NA**
				2	86226	2.00				
				3	63529	1.48				
				4	56908	1.32				
				Mean	**67307**	**1.57**				
			25	1	29136	0.68				
				2	45518	1.06				
				3	42708	0.99				
				4	38074	0.89				
				Mean	**38859**	**0.90**				
			50	1	33511	0.78				
				2	41282	0.96				
				3	36712	0.85				
				4	26023	0.61				
				Mean	**34382**	**0.80**				
4	Vehicle - Positive Control		0	1	61301	1.49				
				2	42018	1.02				
				3	31933	0.78				
				4	29486	0.72				
				Mean	**41184**	**1.00**				
4	Positive Control		NA	1	188993	4.59				
				2	168896	4.10				
				3	258012	6.26				
				4	307187	7.46				
				Mean	**230772**	**5.60**				
4	Vehicle - Substance	AOO	0	1	55245	1.29				
				2	32859	0.77				
				3	37143	0.87				
				4	46219	1.08				
				Mean	**42866**	**1.00**				
4	Isoeugenol	AOO	1	1	117220	2.73	**1.11**	**0.66**	**0.41**	**0.34**
				2	159050	3.71				
				3	114887	2.68				
				4	112197	2.62				
				Mean	**125838**	**2.94**				
			3	1	167018	3.90				
				2	172577	4.03				
				3	190296	4.44				
				4	171216	3.99				
				Mean	**175277**	**4.09**				
			10	1	278270	6.49				
				2	266047	6.21				
				3	212878	4.97				
				4	291279	6.80				
				Mean	**262118**	**6.11**				
4	2,4-Dinitrochloro-benzene	AOO	0.03	1	99433	2.32	**0.03**	**0.02**	**0.02**	**0.01**
				2	124385	2.90				
				3	156964	3.66				
				4	131177	3.06				
				Mean	**127990**	**2.99**				
			0.10	1	239929	5.60				
				2	248752	5.80				
				3	226511	5.28				
				4	125633	2.93				
				Mean	**210206**	**4.90**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
4	2,4-Dinitrochloro-benzene (continued)		0.30	1	351048	8.19				
				2	304028	7.09				
				3	426667	9.95				
				4	381330	8.90				
				Mean	**365768**	**8.53**				
5	Vehicle - Positive Control		0	1	7783	0.65				
				2	7273	0.61				
				3	22835	1.92				
				4	9704	0.82				
				Mean	**11899**	**1.00**				
5	Positive Control		NA	1	60519	5.09				
				2	57983	4.87				
				3	48159	4.05				
				4	72951	6.13				
				Mean	**59903**	**5.03**				
5	Vehicle - Substance	AOO	0	1	31442	1.49				
				2	12103	0.57				
				3	20941	0.99				
				4	20115	0.95				
				Mean	**21150**	**1.00**				
5	2,4-Dinitrochloro-benzene	AOO	0.03	1	19491	0.92	**0.13**	**0.11**	**0.09**	**0.08**
				2	14102	0.67				
				3	17254	0.82				
				4	21584	1.02				
				Mean	**18107**	**0.86**				
			0.10	1	40351	1.91				
				2	76157	3.60				
				3	39813	1.88				
				4	26445	1.25				
				Mean	**45691**	**2.16**				
			0.30	1	199476	9.43				
				2	109134	5.16				
				3	155961	7.37				
				4	200326	9.47				
				Mean	**166224**	**7.86**				
5	Isoeugenol	AOO	1	1	20321	0.96	**5.98**	**5.19**	**4.40**	**4.08**
				2	19512	0.92				
				3	33957	1.61				
				4	17792	0.84				
				Mean	**22896**	**1.08**				
			3	1	12620	0.60				
				2	28001	1.32				
				3	20937	0.99				
				4	32921	1.56				
				Mean	**23619**	**1.12**				
			10	1	123238	5.83				
				2	110582	5.23				
				3	118049	5.58				
				4	116524	5.51				
				Mean	**117098**	**5.54**				
5	Vehicle - Positive Control		0	1	22681	1.23				
				2	15429	0.84				
				3	20405	1.11				
				4	15143	0.82				
				Mean	**18414**	**1.00**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
5	Positive Control		NA	1	97304	5.28				
				2	83132	4.51				
				3	67441	3.66				
				4	117794	6.40				
				Mean	**91418**	**4.96**				
5	Vehicle - Substance	AOO	0	1	16435	0.86				
				2	22909	1.20				
				3	25965	1.36				
				4	11275	0.59				
				Mean	**19146**	**1.00**				
5	Hexyl cinnamic aldehyde	AOO	5	1	17037	0.89	**18.13**	**14.59**	**11.06**	**9.60**
				2	30640	1.60				
				3	26481	1.38				
				4	19509	1.02				
				Mean	**23417**	**1.22**				
			10	1	32966	1.72				
				2	38027	1.99				
				3	17968	0.94				
				4	52769	2.76				
				Mean	**35432**	**1.85**				
			25	1	73109	3.82				
				2	83266	4.35				
				3	77637	4.05				
				4	70103	3.66				
				Mean	**76029**	**3.97**				
5	Isopropanol	AOO	10	1	9967	0.52	**NA**	**NA**	**NA**	**NA**
				2	5679	0.30				
				3	12157	0.63				
				4	12621	0.66				
				Mean	**10106**	**0.53**				
			25	1	15066	0.79				
				2	15418	0.81				
				3	12221	0.64				
				4	15418	0.81				
				Mean	**14531**	**0.76**				
			50	1	18749	0.98				
				2	13502	0.71				
				3	10223	0.53				
				4	11851	0.62				
				Mean	**13581**	**0.71**				
5	Vehicle - Positive Control		0	1	15918	1.04				
				2	13724	0.90				
				3	10819	0.71				
				4	20489	1.34				
				Mean	**15237**	**1.00**				
5	Positive Control		NA	1	67799	4.45				
				2	56834	3.73				
				3	60000	3.94				
				4	84607	5.55				
				Mean	**67310**	**4.42**				
5	Vehicle - Substance	ACE	0	1	8265	0.50				
				2	23012	1.40				
				3	14503	0.88				
				4	19975	1.22				
				Mean	**16439**	**1.00**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
5	Glutaraldehyde	ACE	0.05	1	23621	1.44	**NA**	**0.29**	**0.12**	**0.10**
				2	11837	0.72				
				3	14251	0.87				
				4	18389	1.12				
				Mean	**17024**	**1.04**				
			0.15	1	38622	2.35				
				2	64431	3.92				
				3	24666	1.50				
				4	33558	2.04				
				Mean	**40319**	**2.45**				
			0.50	1	34431	2.09				
				2	42955	2.61				
				3	42380	2.58				
				4	49184	2.99				
				Mean	**42237**	**2.57**				
5	Formaldehyde	ACE	0.5	1	24898	1.51	**NA**	**4.18**	**2.02**	**1.38**
				2	18454	1.12				
				3	21972	1.34				
				4	12719	0.77				
				Mean	**19510**	**1.19**				
			1.5	1	36696	2.23				
				2	29172	1.77				
				3	43949	2.67				
				4	14018	0.85				
				Mean	**30959**	**1.88**				
			5.0	1	44219	2.69				
				2	47739	2.90				
				3	33377	2.03				
				4	51542	3.14				
				Mean	**44219**	**2.69**				
6	Vehicle - Positive Control		0	1	16022	1.79				
				2	9436	1.05				
				3	3788	0.42				
				4	6561	0.73				
				Mean	**8952**	**1.00**				
6	Positive Control		NA	1	80444	8.99				
				2	92491	10.33				
				3	73767	8.24				
				4	101082	11.29				
				Mean	**86946**	**9.71**				
6	Vehicle - Substance	DMSO	0	1	7575	1.81				
				2	4135	0.99				
				3	2759	0.66				
				4	2267	0.54				
				Mean	**4184**	**1.00**				
6	Nickel (II) sulfate hexahydrate	DMSO	1	1	30363	7.26	**0.47**	**0.35**	**0.24**	**0.19**
				2	12902	3.08				
				3	22353	5.34				
				4	22343	5.34				
				Mean	**21990**	**5.26**				
			3	1	32830	7.85				
				2	28614	6.84				
				3	31319	7.49				
				4	19101	4.57				
				Mean	**27966**	**6.68**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
6	Nickel (II) sulfate hexahydrate (continued)		10	1	46902	11.21				
				2	64448	15.40				
				3	56156	13.42				
				4	29707	7.10				
				Mean	**49303**	**11.78**				
6	Cobalt chloride	DMSO	0.3	1	88782	21.22	**0.06**	**0.05**	**0.03**	**0.03**
				2	40452	9.67				
				3	22788	5.45				
				4	23988	5.73				
				Mean	**44002**	**10.52**				
			1.0	1	59079	14.12				
				2	24246	5.80				
				3	69511	16.61				
				4	25023	5.98				
				Mean	**44465**	**10.63**				
			3.0	1	108860	26.02				
				2	62637	14.97				
				3	106164	25.38				
				4	66252	15.84				
				Mean	**85978**	**20.55**				
6	Vehicle - Positive Control		0	1	7997	0.75				
				2	10763	1.01				
				3	13602	1.27				
				4	10360	0.97				
				Mean	**10680**	**1.00**				
6	Positive Control		NA	1	52468	4.91				
				2	66048	6.18				
				3	81979	7.68				
				4	76135	7.13				
				Mean	**69157**	**6.48**				
6	Vehicle - Substance	AOO	0	1	8621	0.62				
				2	14670	1.05				
				3	18086	1.30				
				4	14263	1.03				
				Mean	**13910**	**1.00**				
6	Abietic acid	AOO	5	1	38117	2.74	**7.54**	**6.47**	**5.39**	**4.88**
				2	18850	1.36				
				3	25525	1.83				
				4	18617	1.34				
				Mean	**25277**	**1.82**				
			10	1	57039	4.10				
				2	73842	5.31				
				3	56561	4.07				
				4	43018	3.09				
				Mean	**57615**	**4.14**				
			25	1	98752	7.10				
				2	129426	9.30				
				3	139343	10.02				
				4	75268	5.41				
				Mean	**110697**	**7.96**				
6	2,4-Dinitrochloro-benzene	AOO	0.03	1	29344	2.11	**0.04**	**0.03**	**0.02**	**0.01**
				2	53129	3.82				
				3	39348	2.83				
				4	31167	2.24				
				Mean	**38247**	**2.75**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
6	2,4-Dinitrochloro-benzene (continued)		0.10	1	32064	2.31				
				2	78273	5.63				
				3	66285	4.77				
				4	60587	4.36				
				Mean	**59302**	**4.26**				
			0.30	1	170451	12.25				
				2	258700	18.60				
				3	241703	17.38				
				4	171691	12.34				
				Mean	**210636**	**15.14**				
6	Vehicle - Positive Control		0	1	18240	1.56				
				2	4174	0.36				
				3	11817	1.01				
				4	12605	1.08				
				Mean	**11709**	**1.00**				
6	Positive Control		NA	1	105716	9.03				
				2	92508	7.90				
				3	86410	7.38				
				4	107936	9.22				
				Mean	**98142**	**8.38**				
6	Vehicle - Substance	AOO	0	1	13188	0.81				
				2	16677	1.02				
				3	13789	0.84				
				4	21847	1.33				
				Mean	**16375**	**1.00**				
6	Hexyl cinnamic aldehyde	AOO	5	1	34939	2.13	**13.13**	**10.76**	**7.46**	**5.96**
				2	34548	2.11				
				3	18582	1.13				
				4	21408	1.31				
				Mean	**27369**	**1.67**				
			10	1	50225	3.07				
				2	38763	2.37				
				3	26933	1.64				
				4	37387	2.28				
				Mean	**38327**	**2.34**				
			25	1	61340	3.75				
				2	71280	4.35				
				3	110980	6.78				
				4	116668	7.12				
				Mean	**90067**	**5.50**				
6	Isopropanol	AOO	10	1	71570	4.37	**NA**	**NA**	**NA**	**IDR**
				2	20763	1.27				
				3	19846	1.21				
				4	16753	1.02				
				Mean	**32233**	**1.97**				
			25	1	14610	0.89				
				2	19836	1.21				
				3	17188	1.05				
				4	7416	0.45				
				Mean	**14762**	**0.90**				
			50	1	16623	1.02				
				2	19168	1.17				
				3	28176	1.72				
				4	21474	1.31				
				Mean	**21360**	**1.30**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
7	Vehicle - Positive Control		0	1	10954	0.47				
				2	14547	0.62				
				3	33870	1.44				
				4	34460	1.47				
				Mean	**23458**	**1.00**				
7	Positive Control		NA	1	93512	3.99				
				2	104433	4.45				
				3	114003	4.86				
				4	180482	7.69				
				Mean	**123107**	**5.25**				
7	Vehicle - Substance	AOO	0	1	15339	0.71				
				2	11627	0.54				
				3	17793	0.83				
				4	41425	1.92				
				Mean	**21546**	**1.00**				
7	Methyl salicylate	AOO	5	1	26796	1.24	**NA**	**NA**	**NA**	**NA**
				2	23023	1.07				
				3	12934	0.60				
				4	31083	1.44				
				Mean	**23459**	**1.09**				
			10	1	30066	1.40				
				2	45494	2.11				
				3	41639	1.93				
				4	35433	1.64				
				Mean	**38158**	**1.77**				
			25	1	14218	0.66				
				2	31612	1.47				
				3	31551	1.46				
				4	42145	1.96				
				Mean	**29881**	**1.39**				
7	Abietic acid	AOO	5	1	28706	1.33	**7.68**	**11.53**	**6.33**	**4.60**
				2	46411	2.15				
				3	46541	2.16				
				4	39654	1.84				
				Mean	**40328**	**1.87**				
			10	1	50807	2.36				
				2	92597	4.30				
				3	105497	4.90				
				4	94381	4.38				
				Mean	**85821**	**3.98**				
			25	1	45895	2.13				
				2	102739	4.77				
				3	87409	4.06				
				4	91230	4.23				
				Mean	**81818**	**3.80**				
7	Vehicle - Positive Control		0	1	17271	0.75				
				2	23663	1.03				
				3	24070	1.04				
				4	27154	1.18				
				Mean	**23039**	**1.00**				
7	Positive Control		NA	1	127080	5.52				
				2	150247	6.52				
				3	122132	5.30				
				4	128311	5.57				
				Mean	**131942**	**5.73**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
7	Vehicle - Substance	AOO	0	1	36823	1.23				
				2	31245	1.04				
				3	21937	0.73				
				4	29694	0.99				
				Mean	**29925**	**1.00**				
7	Hexyl cinnamic aldehyde	AOO	5	1	42392	1.42	**7.71**	**6.78**	**5.85**	**5.48**
				2	33988	1.14				
				3	66350	2.22				
				4	41865	1.40				
				Mean	**46148**	**1.54**				
			10	1	106569	3.56				
				2	151880	5.08				
				3	161431	5.39				
				4	87141	2.91				
				Mean	**126755**	**4.24**				
			25	1	170985	5.71				
				2	193134	6.45				
				3	198620	6.64				
				4	286402	9.57				
				Mean	**212285**	**7.09**				
7	Isopropanol	AOO	10	1	30442	1.02	**NA**	**NA**	**NA**	**NA**
				2	32600	1.09				
				3	41239	1.38				
				4	69502	2.32				
				Mean	**43446**	**1.45**				
			25	1	15392	0.51				
				2	39028	1.30				
				3	22387	0.75				
				4	32333	1.08				
				Mean	**27285**	**0.91**				
			50	1	26039	0.87				
				2	25885	0.87				
				3	27685	0.93				
				4	19497	0.65				
				Mean	**24776**	**0.83**				
7	Vehicle - Positive Control		0	1	20353	0.71				
				2	31709	1.10				
				3	34254	1.19				
				4	29038	1.01				
				Mean	**28838**	**1.00**				
7	Positive Control		NA	1	170163	5.90				
				2	142824	4.95				
				3	167113	5.79				
				4	135621	4.70				
				Mean	**153930**	**5.34**				
7	Vehicle - Substance	AOO	0	1	25299	1.13				
				2	25685	1.14				
				3	19870	0.88				
				4	19010	0.85				
				Mean	**22466**	**1.00**				
7	Dimethyl isophthalate	AOO	5	1	30872	1.37	**NA**	**NA**	**NA**	**NA**
				2	23829	1.06				
				3	26046	1.16				
				4	32477	1.45				
				Mean	**28306**	**1.26**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
7	Dimethyl isophthalate (continued)		10	1	28765	1.28				
				2	27567	1.23				
				3	22517	1.00				
				4	23373	1.04				
				Mean	**25555**	**1.14**				
			25	1	24457	1.09				
				2	25583	1.14				
				3	18065	0.80				
				4	26228	1.17				
				Mean	**23583**	**1.05**				
7	2,4-Dinitrochloro-benzene	AOO	0.03	1	54379	2.42	**0.02**	**0.01**	**0.01**	**0.01**
				2	95575	4.25				
				3	95094	4.23				
				4	99284	4.42				
				Mean	**86083**	**3.83**				
			0.10	1	142045	6.32				
				2	139187	6.20				
				3	108882	4.85				
				4	93969	4.18				
				Mean	**121021**	**5.39**				
			0.30	1	282805	12.59				
				2	336813	14.99				
				3	258764	11.52				
				4	305713	13.61				
				Mean	**296024**	**13.18**				
8	Vehicle - Positive Control		0	1	18303	0.95				
				2	25980	1.34				
				3	17493	0.90				
				4	15606	0.81				
				Mean	**19345**	**1.00**				
8	Positive Control		NA	1	98761	5.11				
				2	72937	3.77				
				3	86236	4.46				
				4	76278	3.94				
				Mean	**83553**	**4.32**				
8	Vehicle - Substance	AOO	0	1	9463	0.78				
				2	13874	1.14				
				3	17229	1.41				
				4	8262	0.68				
				Mean	**12207**	**1.00**				
8	Isopropanol	AOO	10	1	12562	1.03	**NA**	**NA**	**NA**	**NA**
				2	17330	1.42				
				3	11886	0.97				
				4	17410	1.43				
				Mean	**14797**	**1.21**				
			25	1	17249	1.41				
				2	9264	0.76				
				3	11845	0.97				
				4	11193	0.92				
				Mean	**12387**	**1.01**				
			50	1	14510	1.19				
				2	14113	1.16				
				3	12238	1.00				
				4	13342	1.09				
				Mean	**13551**	**1.11**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
8	Hexyl cinnamic aldehyde	AOO	5	1	16997	1.39	**7.92**	**7.03**	**6.14**	**5.78**
				2	15777	1.29				
				3	22473	1.84				
				4	11217	0.92				
				Mean	**16616**	**1.36**				
			10	1	40975	3.36				
				2	56754	4.65				
				3	58346	4.78				
				4	47242	3.87				
				Mean	**50829**	**4.16**				
			25	1	155208	12.71				
				2	133055	10.90				
				3	75582	6.19				
				4	135369	11.09				
				Mean	**124803**	**10.22**				
8	Vehicle - Positive Control		0	1	11818	0.62				
				2	22893	1.19				
				3	21441	1.12				
				4	20608	1.07				
				Mean	**19190**	**1.00**				
8	Positive Control		NA	1	117067	6.10				
				2	100222	5.22				
				3	91462	4.77				
				4	80907	4.22				
				Mean	**97414**	**5.08**				
8	Vehicle - Substance	DMSO	0	1	15322	0.77				
				2	24630	1.24				
				3	16802	0.85				
				4	22460	1.13				
				Mean	**19803**	**1.00**				
8	Nickel (II) sulfate hexahydrate	DMSO	1	1	64139	3.24	**IDR**	**IDR**	**IDR**	**IDR**
				2	59705	3.01				
				3	61654	3.11				
				4	90810	4.59				
				Mean	**69077**	**3.49**				
			3	1	64301	3.25				
				2	70343	3.55				
				3	55459	2.80				
				4	53420	2.70				
				Mean	**60881**	**3.07**				
			10	1	40447	2.04				
				2	45033	2.27				
				3	62589	3.16				
				4	54206	2.74				
				Mean	**50568**	**2.55**				
8	Cobalt chloride	DMSO	0.3	1	68800	3.47	**0.14**	**0.10**	**0.08**	**0.07**
				2	98124	4.95				
				3	95925	4.84				
				4	87399	4.41				
				Mean	**87562**	**4.42**				
			1.0	1	123857	6.25				
				2	178916	9.03				
				3	96477	4.87				
				4	124765	6.30				
				Mean	**131004**	**6.62**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
8	Cobalt chloride (continued)		3.0	1	175242	8.85				
				2	143477	7.25				
				3	155827	7.87				
				4	164687	8.32				
				Mean	**159808**	**8.07**				
8	Vehicle - Positive Control		0	1	17139	1.02				
				2	23311	1.39				
				3	14001	0.84				
				4	12548	0.75				
				Mean	**16749**	**1.00**				
8	Positive Control		NA	1	133873	7.99				
				2	147108	8.78				
				3	114171	6.82				
				4	97568	5.83				
				Mean	**123180**	**7.35**				
8	Vehicle - Substance	AOO	0	1	18744	0.91				
				2	20074	0.98				
				3	15187	0.74				
				4	28298	1.38				
				Mean	**20576**	**1.00**				
8	2,4-Dinitrochloro-benzene	AOO	0.03	1	40777	1.98	**0.10**	**0.04**	**0.02**	**0.02**
				2	45024	2.19				
				3	30526	1.48				
				4	82593	4.01				
				Mean	**49730**	**2.42**				
			0.10	1	41930	2.04				
				2	50135	2.44				
				3	107465	5.22				
				4	50754	2.47				
				Mean	**62571**	**3.04**				
			0.30	1	228871	11.12				
				2	393845	19.14				
				3	273309	13.28				
				4	140789	6.84				
				Mean	**259203**	**12.60**				
8	3-Aminophenol	AOO	1	1	25653	1.25	**NA**	**NA**	**3.18**	**2.51**
				2	27127	1.32				
				3	28861	1.40				
				4	19026	0.92				
				Mean	**25167**	**1.22**				
			3	1	51618	2.51				
				2	47941	2.33				
				3	36281	1.76				
				4	27846	1.35				
				Mean	**40921**	**1.99**				
			10	1	57296	2.78				
				2	52938	2.57				
				3	38134	1.85				
				4	47782	2.32				
				Mean	**49037**	**2.38**				
9	Vehicle - Positive Control		0	1	25729	0.98				
				2	31786	1.22				
				3	24343	0.93				
				4	22785	0.87				
				Mean	**26161**	**1.00**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
9	Positive Control		NA	1	155962	5.96				
				2	112682	4.31				
				3	124334	4.75				
				4	122066	4.67				
				Mean	**128761**	**4.92**				
9	Vehicle - Substance	AOO	0	1	21600	0.73				
				2	38136	1.29				
				3	34690	1.17				
				4	23981	0.81				
				Mean	**29602**	**1.00**				
9	Hexyl cinnamic aldehyde	AOO	5	1	35263	1.19	**17.07**	**12.55**	**9.19**	**8.46**
				2	34558	1.17				
				3	20309	0.69				
				4	12277	0.41				
				Mean	**25602**	**0.86**				
			10	1	32104	1.08				
				2	68901	2.33				
				3	61583	2.08				
				4	99972	3.38				
				Mean	**65640**	**2.22**				
			25	1	109826	3.71				
				2	114755	3.88				
				3	101116	3.42				
				4	133469	4.51				
				Mean	**114791**	**3.88**				
9	Isopropanol	AOO	10	1	16071	0.54	**NA**	**NA**	**NA**	**NA**
				2	29909	1.01				
				3	16721	0.56				
				4	12462	0.42				
				Mean	**18791**	**0.63**				
			25	1	18605	0.63				
				2	12916	0.44				
				3	26806	0.91				
				4	24183	0.82				
				Mean	**20627**	**0.70**				
			50	1	11350	0.38				
				2	14836	0.50				
				3	13840	0.47				
				4	20129	0.68				
				Mean	**15039**	**0.51**				
9	Vehicle - Positive Control		0	1	21626	0.82				
				2	28191	1.06				
				3	36208	1.37				
				4	19953	0.75				
				Mean	**26494**	**1.00**				
9	Positive Control		NA	1	152153	5.74				
				2	173639	6.55				
				3	117177	4.42				
				4	165097	6.23				
				Mean	**152016**	**5.74**				
9	Vehicle - Substance	AOO	0	1	37188	1.39				
				2	20177	0.75				
				3	17473	0.65				
				4	32530	1.21				
				Mean	**26842**	**1.00**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
9	Isoeugenol	AOO	1	1	43063	1.60	**2.30**	**0.87**	**0.38**	**0.27**
				2	92318	3.44				
				3	73315	2.73				
				4	68329	2.55				
				Mean	**69256**	**2.58**				
			3	1	82412	3.07				
				2	114677	4.27				
				3	83819	3.12				
				4	65486	2.44				
				Mean	**86598**	**3.23**				
			10	1	241256	8.99				
				2	169293	6.31				
				3	153506	5.72				
				4	197513	7.36				
				Mean	**190392**	**7.09**				
9	2,4-Dinitrochloro-benzene	AOO	0.03	1	80731	3.01	**0.04**	**0.02**	**0.02**	**0.01**
				2	46072	1.72				
				3	82472	3.07				
				4	91886	3.42				
				Mean	**75290**	**2.80**				
			0.10	1	81426	3.03				
				2	105837	3.94				
				3	164718	6.14				
				4	97148	3.62				
				Mean	**112282**	**4.18**				
			0.30	1	294486	10.97				
				2	287848	10.72				
				3	287739	10.72				
				4	298846	11.13				
				Mean	**292230**	**10.89**				
10	Vehicle - Positive Control		0	1	20162	0.95				
				2	15285	0.72				
				3	30517	1.43				
				4	19166	0.90				
				Mean	**21282**	**1.00**				
10	Positive Control		NA	1	116157	5.46				
				2	142905	6.71				
				3	135316	6.36				
				4	117862	5.54				
				Mean	**128060**	**6.02**				
10	Vehicle - Substance	AOO	0	1	45394	0.85				
				2	67917	1.27				
				3	36479	0.68				
				4	63610	1.19				
				Mean	**53350**	**1.00**				
10	2,4-Dinitrochloro-benzene	AOO	0.03	1	52123	0.98	**0.17**	**0.13**	**0.09**	**0.08**
				2	66363	1.24				
				3	36583	0.69				
				4	92933	1.74				
				Mean	**62000**	**1.16**				
			0.10	1	113324	2.12				
				2	80089	1.50				
				3	127648	2.39				
				4	127592	2.39				
				Mean	**112163**	**2.10**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
10	2,4-Dinitrochloro-benzene (continued)		0.30	1	202245	3.79				
				2	264292	4.95				
				3	298490	5.59				
				4	239662	4.49				
				Mean	**251172**	**4.71**				
10	Methyl salicylate	AOO	5	1	36446	0.68	**NA**	**NA**	**NA**	**NA**
				2	34905	0.65				
				3	37286	0.70				
				4	26017	0.49				
				Mean	**33663**	**0.63**				
			10	1	47420	0.89				
				2	47616	0.89				
				3	40117	0.75				
				4	31641	0.59				
				Mean	**41698**	**0.78**				
			25	1	53941	1.01				
				2	54989	1.03				
				3	43082	0.81				
				4	25692	0.48				
				Mean	**44426**	**0.83**				
10	Vehicle - Positive Control		0	1	20445	0.88				
				2	15079	0.65				
				3	26464	1.13				
				4	31358	1.34				
				Mean	**23336**	**1.00**				
10	Positive Control		NA	1	89914	3.85				
				2	107768	4.62				
				3	93418	4.00				
				4	102331	4.39				
				Mean	**98357**	**4.21**				
10	Vehicle - Substance	AOO	0	1	28181	0.97				
				2	33325	1.15				
				3	27821	0.96				
				4	26981	0.93				
				Mean	**29077**	**1.00**				
10	Hexyl cinnamic aldehyde	AOO	5	1	35684	1.23	**15.24**	**9.14**	**7.26**	**6.51**
				2	30080	1.03				
				3	62393	2.15				
				4	34584	1.19				
				Mean	**40685**	**1.40**				
			10	1	86735	2.98				
				2	88833	3.06				
				3	75607	2.60				
				4	66109	2.27				
				Mean	**79321**	**2.73**				
			25	1	78538	2.70				
				2	107305	3.69				
				3	129081	4.44				
				4	93013	3.20				
				Mean	**101984**	**3.51**				
10	Isopropanol	AOO	10	1	19691	0.68	**NA**	**NA**	**NA**	**NA**
				2	28293	0.97				
				3	29845	1.03				
				4	28091	0.97				
				Mean	**26480**	**0.91**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
10	Isopropanol (continued)		25	1	30241	1.04				
				2	24774	0.85				
				3	29230	1.01				
				4	38461	1.32				
				Mean	**30676**	**1.06**				
			50	1	42188	1.45				
				2	37228	1.28				
				3	35247	1.21				
				4	30201	1.04				
				Mean	**36216**	**1.25**				
11	Vehicle - Positive Control		0	1	13452	0.45				
				2	32469	1.09				
				3	37235	1.25				
				4	35940	1.21				
				Mean	**29774**	**1.00**				
11	Positive Control		NA	1	113708	3.82				
				2	108755	3.65				
				3	57560	1.93				
				4	97736	3.28				
				Mean	**94440**	**3.17**				
11	Vehicle - Substance	AOO	0	1	16175	0.76				
				2	31955	1.50				
				3	24257	1.14				
				4	12926	0.61				
				Mean	**21328**	**1.00**				
11	Hexyl cinnamic aldehyde	AOO	5	1	24541	1.15	**9.13**	**7.74**	**6.35**	**5.79**
				2	31920	1.50				
				3	42454	1.99				
				4	30308	1.42				
				Mean	**32306**	**1.51**				
			10	1	73959	3.47				
				2	73920	3.47				
				3	74762	3.51				
				4	60117	2.82				
				Mean	**70689**	**3.31**				
			25	1	56324	2.64				
				2	81323	3.81				
				3	117271	5.50				
				4	126476	5.93				
				Mean	**95348**	**4.47**				
11	Vehicle - Positive Control		0	1	6855	0.32				
				2	23315	1.10				
				3	27767	1.30				
				4	27187	1.28				
				Mean	**21281**	**1.00**				
11	Positive Control		NA	1	118741	5.58				
				2	114600	5.39				
				3	86525	4.07				
				4	115969	5.45				
				Mean	**108959**	**5.12**				
11	Vehicle - Substance	DMSO	0	1	67859	1.04				
				2	76567	1.18				
				3	60349	0.93				
				4	55465	0.85				
				Mean	**65060**	**1.00**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
11	Potassium dichromate	DMSO	0.1	1	134992	2.07	**0.51**	**0.37**	**0.16**	**0.09**
				2	133187	2.05				
				3	130433	2.00				
				4	97134	1.49				
				Mean	**123936**	**1.90**				
			0.3	1	194686	2.99				
				2	104933	1.61				
				3	166086	2.55				
				4	117627	1.81				
				Mean	**145833**	**2.24**				
			1.0	1	283541	4.36				
				2	340279	5.23				
				3	318543	4.90				
				4	301673	4.64				
				Mean	**311009**	**4.78**				
11	Lactic acid	DMSO	5	1	34889	0.54	**NA**	**NA**	**NA**	**NA**
				2	70275	1.08				
				3	81876	1.26				
				4	55263	0.85				
				Mean	**60576**	**0.93**				
			10	1	57810	0.89				
				2	60103	0.92				
				3	42148	0.65				
				4	36073	0.55				
				Mean	**49033**	**0.75**				
			25	1	73850	1.14				
				2	38479	0.59				
				3	54647	0.84				
				4	41547	0.64				
				Mean	**52131**	**0.80**				
11	Vehicle - Positive Control		0	1	25338	0.96				
				2	29261	1.11				
				3	21131	0.80				
				4	29732	1.13				
				Mean	**26365**	**1.00**				
11	Positive Control		NA	1	136936	5.19				
				2	81100	3.08				
				3	114598	4.35				
				4	79191	3.00				
				Mean	**102956**	**3.90**				
11	Vehicle - Substance	DMSO	0	1	86043	1.05				
				2	65589	0.80				
				3	117592	1.43				
				4	59151	0.72				
				Mean	**82093**	**1.00**				
11	Cobalt chloride	DMSO	1	1	113621	1.38	**NA**	**NA**	**4.93**	**3.5**
				2	130468	1.59				
				3	97082	1.18				
				4	147603	1.80				
				Mean	**122193**	**1.49**				
			3	1	123437	1.50				
				2	115859	1.41				
				3	189281	2.31				
				4	139101	1.69				
				Mean	**141919**	**1.73**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
11	Cobalt chloride (continued)		5	1	167985	2.05				
				2	167593	2.04				
				3	174922	2.13				
				4	150902	1.84				
				Mean	**165350**	**2.01**				
11	Nickel (II) sulfate hexahydrate	DMSO	1	1	65339	0.80	**NA**	**NA**	**NA**	**NA**
				2	51981	0.63				
				3	46829	0.57				
				4	50461	0.61				
				Mean	**53652**	**0.65**				
			3	1	89247	1.09				
				2	49391	0.60				
				3	83879	1.02				
				4	37620	0.46				
				Mean	**65034**	**0.79**				
			10	1	80662	0.98				
				2	49864	0.61				
				3	41820	0.51				
				4	69460	0.85				
				Mean	**60451**	**0.74**				
12	Vehicle - Positive Control		0	1	31062	1.15				
				2	34769	1.28				
				3	19233	0.71				
				4	23272	0.86				
				Mean	**27084**	**1.00**				
12	Positive Control		NA	1	32499	1.20				
				2	149284	5.51				
				3	138062	5.10				
				4	155617	5.75				
				Mean	**118865**	**4.39**				
12	Vehicle - Substance	AOO	0	1	34707	1.27				
				2	19823	0.72				
				3	21963	0.80				
				4	33252	1.21				
				Mean	**27436**	**1.00**				
12	Hexyl cinnamic aldehyde	AOO	5	1	45866	1.67	**8.76**	**7.37**	**5.98**	**5.43**
				2	32444	1.18				
				3	52964	1.93				
				4	49440	1.80				
				Mean	**45178**	**1.65**				
			10	1	96208	3.51				
				2	70432	2.57				
				3	121167	4.42				
				4	90169	3.29				
				Mean	**94494**	**3.44**				
			25	1	146684	5.35				
				2	176112	6.42				
				3	135063	4.92				
				4	168604	6.15				
				Mean	**156615**	**5.71**				
12	Vehicle - Positive Control		0	1	26207	0.79				
				2	39177	1.18				
				3	37398	1.13				
				4	30062	0.91				
				Mean	**33211**	**1.00**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
12	Positive Control		NA	1	151987	4.58				
				2	169589	5.11				
				3	209928	6.32				
				4	134469	4.05				
				Mean	**166493**	**5.01**				
12	Vehicle - Substance	DMSO	0	1	78629	0.95				
				2	88765	1.07				
				3	76637	0.92				
				4	88155	1.06				
				Mean	**83046**	**1.00**				
12	Nickel (II) sulfate hexahydrate	DMSO	1	1	98797	1.19	**NA**	**NA**	**NA**	**NA**
				2	80665	0.97				
				3	86949	1.05				
				4	65175	0.78				
				Mean	**82896**	**1.00**				
			3	1	84327	1.02				
				2	86877	1.05				
				3	137747	1.66				
				4	104430	1.26				
				Mean	**103345**	**1.24**				
			10	1	105221	1.27				
				2	71971	0.87				
				3	55567	0.67				
				4	89624	1.08				
				Mean	**80596**	**0.97**				
12	Potassium dichromate	DMSO	0.1	1	170554	2.05	**0.49**	**0.27**	**0.13**	**0.09**
				2	113710	1.37				
				3	166200	2.00				
				4	179394	2.16				
				Mean	**157464**	**1.90**				
			0.3	1	198199	2.39				
				2	205018	2.47				
				3	273194	3.29				
				4	191835	2.31				
				Mean	**217061**	**2.61**				
			1.0	1	301077	3.63				
				2	323900	3.90				
				3	378405	4.56				
				4	351057	4.23				
				Mean	**338610**	**4.08**				
13	Vehicle - Positive Control		0	1	21808	0.80				
				2	23919	0.87				
				3	24606	0.90				
				4	39312	1.43				
				Mean	**27411**	**1.00**				
13	Positive Control		NA	1	138513	5.05				
				2	94225	3.44				
				3	118316	4.32				
				4	161413	5.89				
				Mean	**128117**	**4.67**				
13	Vehicle - Substance	AOO	0	1	33895	1.37				
				2	20013	0.81				
				3	20945	0.85				
				4	24103	0.97				
				Mean	**24739**	**1.00**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
13	Hexyl cinnamic aldehyde	AOO	5	1	28705	1.16	**7.59**	**6.77**	**5.95**	**5.63**
				2	19630	0.79				
				3	45958	1.86				
				4	45943	1.86				
				Mean	**35059**	**1.42**				
			10	1	106862	4.32				
				2	92835	3.75				
				3	83026	3.36				
				4	159832	6.46				
				Mean	**110638**	**4.47**				
			25	1	164960	6.67				
				2	116945	4.73				
				3	118296	4.78				
				4	135132	5.46				
				Mean	**133833**	**5.41**				
13	Vehicle - Positive Control		0	1	16810	0.75				
				2	25921	1.15				
				3	21544	0.96				
				4	25627	1.14				
				Mean	**22475**	**1.00**				
13	Positive Control		NA	1	156378	6.96				
				2	133906	5.96				
				3	140685	6.26				
				4	152161	6.77				
				Mean	**145782**	**6.49**				
13	Vehicle - Substance	DMSO	0	1	93878	1.15				
				2	70631	0.87				
				3	91822	1.13				
				4	68974	0.85				
				Mean	**81326**	**1.00**				
13	Cobalt chloride	DMSO	1	1	120105	1.48	**NA**	**4.13**	**1.88**	**1.38**
				2	148835	1.83				
				3	93820	1.15				
				4	172802	2.12				
				Mean	**133890**	**1.65**				
			3	1	199869	2.46				
				2	195046	2.40				
				3	207281	2.55				
				4	195145	2.40				
				Mean	**199335**	**2.45**				
			5	1	192357	2.37				
				2	215391	2.65				
				3	224902	2.77				
				4	192928	2.37				
				Mean	**206394**	**2.54**				
13	Lactic acid	DMSO	5	1	71011	0.87	**NA**	**NA**	**NA**	**NA**
				2	58742	0.72				
				3	95883	1.18				
				4	96922	1.19				
				Mean	**80639**	**0.99**				
			10	1	58052	0.71				
				2	44480	0.55				
				3	56725	0.70				
				4	62219	0.77				
				Mean	**55369**	**0.68**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
13	Lactic acid (continued)		25	1	61451	0.76				
				2	47962	0.59				
				3	79235	0.97				
				4	51848	0.64				
				Mean	**60124**	**0.74**				
14	Vehicle - Positive Control		0	1	25953	0.86				
				2	42071	1.39				
				3	22870	0.76				
				4	30199	1.00				
				Mean	**30273**	**1.00**				
14	Positive Control		NA	1	198381	6.55				
				2	164826	5.44				
				3	205542	6.79				
				4	198361	6.55				
				Mean	**191777**	**6.33**				
14	Vehicle - Substance	AOO	0	1	21623	0.89				
				2	27737	1.14				
				3	33618	1.38				
				4	14415	0.59				
				Mean	**24348**	**1.00**				
14	Hexyl cinnamic aldehyde	AOO	5	1	45466	1.87	**7.94**	**6.36**	**4.85**	**4.44**
				2	40112	1.65				
				3	72779	2.99				
				4	43275	1.78				
				Mean	**50408**	**2.07**				
			10	1	100580	4.13				
				2	134453	5.52				
				3	18994	0.78				
				4	101713	4.18				
				Mean	**88935**	**3.65**				
			25	1	164791	6.77				
				2	155059	6.37				
				3	249145	10.23				
				4	171572	7.05				
				Mean	**185142**	**7.60**				
14	Vehicle - Positive Control		0	1	18024	0.74				
				2	24615	1.02				
				3	28493	1.18				
				4	25735	1.06				
				Mean	**24216**	**1.00**				
14	Positive Control		NA	1	116341	4.80				
				2	213773	8.83				
				3	182037	7.52				
				4	192821	7.96				
				Mean	**176243**	**7.28**				
14	Vehicle - Substance	DMSO	0	1	33858	0.81				
				2	31373	0.75				
				3	60046	1.44				
				4	41804	1.00				
				Mean	**41770**	**1.00**				
14	Cobalt chloride	DMSO	1	1	104955	2.51	**1.76**	**1.20**	**0.82**	**0.72**
				2	83477	2.00				
				3	85107	2.04				
				4	114867	2.75				
				Mean	**97101**	**2.32**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
14	Cobalt chloride (continued)		3	1	193202	4.63				
				2	147696	3.54				
				3	165128	3.95				
				4	179062	4.29				
				Mean	**171272**	**4.10**				
			5	1	239096	5.72				
				2	128719	3.08				
				3	160037	3.83				
				4	182970	4.38				
				Mean	**177705**	**4.25**				
14	Nickel (II) sulfate hexahydrate	DMSO	1	1	104492	2.50	**NA**	**NA**	**8.40**	**5.94**
				2	58854	1.41				
				3	94853	2.27				
				4	53019	1.27				
				Mean	**77804**	**1.86**				
			3	1	72152	1.73				
				2	48034	1.15				
				3	68084	1.63				
				4	72530	1.74				
				Mean	**65200**	**1.56**				
			10	1	71690	1.72				
				2	NA	NA				
				3	97605	2.34				
				4	97675	2.34				
				Mean	**88990**	**2.13**				
15	Vehicle - Positive Control		0	1	39487	1.12				
				2	45663	1.30				
				3	28492	0.81				
				4	26819	0.76				
				Mean	**35115**	**1.00**				
15	Positive Control		NA	1	157090	4.47				
				2	164583	4.69				
				3	77120	2.20				
				4	157960	4.50				
				Mean	**139188**	**3.96**				
15	Vehicle - Substance	AOO	0	1	26758	0.86				
				2	46603	1.49				
				3	23061	0.74				
				4	28334	0.91				
				Mean	**31189**	**1.00**				
15	Hexyl cinnamic aldehyde	AOO	5	1	38890	1.25	**15.18**	**9.92**	**7.45**	**6.47**
				2	55784	1.79				
				3	43619	1.40				
				4	49120	1.57				
				Mean	**46853**	**1.50**				
			10	1	71984	2.31				
				2	66130	2.12				
				3	84295	2.70				
				4	91478	2.93				
				Mean	**78471**	**2.52**				
			25	1	124344	3.99				
				2	85306	2.74				
				3	142287	4.56				
				4	136649	4.38				
				Mean	**122146**	**3.92**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
15	Vehicle - Positive Control		0	1	43807	1.36				
				2	26515	0.82				
				3	29210	0.90				
				4	29709	0.92				
				Mean	**32310**	**1.00**				
15	Positive Control		NA	1	118146	3.66				
				2	172004	5.32				
				3	135989	4.21				
				4	163682	5.07				
				Mean	**147455**	**4.56**				
15	Vehicle - Substance	DMSO	0	1	35762	0.72				
				2	32858	0.67				
				3	49385	1.00				
				4	79406	1.61				
				Mean	**49353**	**1.00**				
15	Lactic acid	DMSO	5	1	35838	0.73	**NA**	**NA**	**NA**	**NA**
				2	46572	0.94				
				3	43793	0.89				
				4	56717	1.15				
				Mean	**45730**	**0.93**				
			10	1	40908	0.83				
				2	44335	0.90				
				3	70146	1.42				
				4	36323	0.74				
				Mean	**47928**	**0.97**				
			25	1	31906	0.65				
				2	37990	0.77				
				3	33696	0.68				
				4	37444	0.76				
				Mean	**35259**	**0.71**				
15	Potassium dichromate	DMSO	0.1	1	121714	2.47	**0.16**	**0.09**	**0.06**	**0.05**
				2	177882	3.60				
				3	132281	2.68				
				4	93102	1.89				
				Mean	**131244**	**2.66**				
			0.3	1	215997	4.38				
				2	210129	4.26				
				3	226134	4.58				
				4	115017	2.33				
				Mean	**191819**	**3.89**				
			1.0	1	360162	7.30				
				2	191584	3.88				
				3	340917	6.91				
				4	293061	5.94				
				Mean	**296431**	**6.01**				
16	Vehicle - Positive Control		0	1	40980	1.14				
				2	29750	0.83				
				3	37809	1.05				
				4	35687	0.99				
				Mean	**36056**	**1.00**				
16	Positive Control		NA	1	166596	4.62				
				2	324494	9.00				
				3	309550	8.59				
				4	255550	7.09				
				Mean	**264047**	**7.32**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
16	Vehicle - Substance	AOO	0	1	28428	1.00				
				2	25378	0.89				
				3	40570	1.43				
				4	19307	0.68				
				Mean	**28421**	**1.00**				
16	Hexyl cinnamic aldehyde	AOO	5	1	68037	2.39	**6.23**	**5.36**	**4.66**	**4.44**
				2	75307	2.65				
				3	70208	2.47				
				4	47285	1.66				
				Mean	**65209**	**2.29**				
			10	1	134273	4.72				
				2	132074	4.65				
				3	192936	6.79				
				4	127598	4.49				
				Mean	**146720**	**5.16**				
			25	1	255545	8.99				
				2	274377	9.65				
				3	235997	8.30				
				4	190963	6.72				
				Mean	**239220**	**8.42**				
16	Vehicle - Positive Control		0	1	45989	1.19				
				2	31080	0.80				
				3	40234	1.04				
				4	37535	0.97				
				Mean	**38709**	**1.00**				
16	Positive Control		NA	1	266865	6.89				
				2	266443	6.88				
				3	291111	7.52				
				4	264989	6.85				
				Mean	**272352**	**7.04**				
16	Vehicle - Substance	DMSO	0	1	78052	1.02				
				2	111835	1.47				
				3	43088	0.57				
				4	71636	0.94				
				Mean	**76153**	**1.00**				
16	Nickel (II) sulfate hexahydrate	DMSO	1	1	104880	1.38	**NA**	**NA**	**NA**	**NA**
				2	80888	1.06				
				3	92663	1.22				
				4	81686	1.07				
				Mean	**90029**	**1.18**				
			3	1	109460	1.44				
				2	116987	1.54				
				3	110261	1.45				
				4	139021	1.83				
				Mean	**118932**	**1.56**				
			10	1	78555	1.03				
				2	115405	1.52				
				3	88420	1.16				
				4	71548	0.94				
				Mean	**88482**	**1.16**				
16	Lactic acid	DMSO	5	1	56025	0.74	**NA**	**NA**	**NA**	**NA**
				2	72079	0.95				
				3	58768	0.77				
				4	90115	1.18				
				Mean	**69247**	**0.91**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
16	Lactic acid (continued)		10	1	44029	0.58				
				2	67039	0.88				
				3	63161	0.83				
				4	68256	0.90				
				Mean	**60621**	**0.80**				
			25	1	72313	0.95				
				2	47618	0.63				
				3	75699	0.99				
				4	80804	1.06				
				Mean	**69108**	**0.91**				
17	Vehicle - Positive Control		0	1	16598	1.00				
				2	21167	1.28				
				3	20244	1.22				
				4	8376	0.50				
				Mean	**16596**	**1.00**				
17	Positive Control		NA	1	130759	7.88				
				2	159307	9.60				
				3	101692	6.13				
				4	105306	6.35				
				Mean	**124266**	**7.49**				
17	Vehicle - Substance	AOO	0	1	22001	0.92				
				2	17205	0.72				
				3	38937	1.63				
				4	17407	0.73				
				Mean	**23888**	**1.00**				
17	Hexyl cinnamic aldehyde	AOO	5	1	37307	1.56	**7.54**	**6.78**	**6.02**	**5.72**
				2	23097	0.97				
				3	33287	1.39				
				4	32984	1.38				
				Mean	**31668**	**1.33**				
			10	1	96209	4.03				
				2	106660	4.47				
				3	109225	4.57				
				4	129230	5.41				
				Mean	**110331**	**4.62**				
			25	1	123470	5.17				
				2	144993	6.07				
				3	191859	8.03				
				4	156101	6.53				
				Mean	**154106**	**6.45**				
17	Vehicle - Positive Control		0	1	11526	0.63				
				2	12942	0.71				
				3	16830	0.92				
				4	31658	1.74				
				Mean	**18239**	**1.00**				
17	Positive Control		NA	1	152686	8.37				
				2	167020	9.16				
				3	133016	7.29				
				4	160607	8.81				
				Mean	**153332**	**8.41**				
17	Vehicle - Substance	DMSO	0	1	47192	0.93				
				2	45146	0.89				
				3	57466	1.13				
				4	53459	1.05				
				Mean	**50815**	**1.00**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
17	Cobalt chloride	DMSO	1	1	134969	2.66	**1.11**	**0.70**	**0.46**	**0.39**
				2	249468	4.91				
				3	104002	2.05				
				4	106668	2.10				
				Mean	**148776**	**2.93**				
			3	1	206718	4.07				
				2	243849	4.80				
				3	212124	4.17				
				4	201772	3.97				
				Mean	**216116**	**4.25**				
			5	1	297901	5.86				
				2	231316	4.55				
				3	192465	3.79				
				4	306231	6.03				
				Mean	**256978**	**5.06**				
17	Potassium dichromate	DMSO	0.1	1	212537	4.18	**0.09**	**0.06**	**0.05**	**0.04**
				2	192220	3.78				
				3	110195	2.17				
				4	146041	2.87				
				Mean	**165248**	**3.25**				
			0.3	1	281536	5.54				
				2	284296	5.59				
				3	229749	4.52				
				4	232971	4.58				
				Mean	**257138**	**5.06**				
			1.0	1	349431	6.88				
				2	269795	5.31				
				3	278313	5.48				
				4	397799	7.83				
				Mean	**323834**	**6.37**				

Abbreviations: ACE = acetone; Anim. = animal; AOO = acetone: olive oil (4:1); ATP = adenosine triphosphate; Calc. = calculated; Conc. = concentration; DMSO = dimethyl sulfoxide; EC3 = estimated concentration needed to produce a stimulation index of 3; EC2.5 = estimated concentration needed to produce a stimulation index of 2.5; EC2 = estimated concentration needed to produce a stimulation index of 2; EC1.8 = estimated concentration needed to produce a stimulation index of 1.8; IDR = insufficient dose response; NA = not applicable; No. = number; SI = stimulation index; Veh. = vehicle.

[1] Original laboratory records with individual animal data from the LLNA: DA two-phased interlaboratory validation study (Omori et al. 2008) were provided by Study Director Takashi Omori from the Kyoto University School of Public Health in Kyoto, Japan.

[2] Laboratories 1 – 10 participated in the first phase, and laboratories 11 – 17 participated in the second phase of the two-phased interlaboratory validation study.

[3] Two ATP measurements were taken for each animal and the mean ATP is indicated.

[4] EC3 value was calculated based on interpolation or extrapolation formulas discussed in Gerberick et al. 2004.

[5] EC value (i.e., EC2.5, EC2, or EC1.8) was calculated based on modified interpolation or extrapolation formulas for EC3 discussed in Gerberick et al. 2004.

Annex V

Accuracy Analyses Using Additional Approaches for Combining Multiple Test Results

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1.0 Accuracy Analyses Using Alternative Decision Criteria and Alternate Methods for Combining Data for Substances Tested Multiple Times

This annex shows performance analyses for the murine local lymph node assay (LLNA) modified by Daicel Chemical Industries, Ltd., based on ATP content (referred to hereafter as the "LLNA: DA") for alternative decision criteria when using two different approaches for combining test results for the 14 substances with multiple LLNA: DA tests.

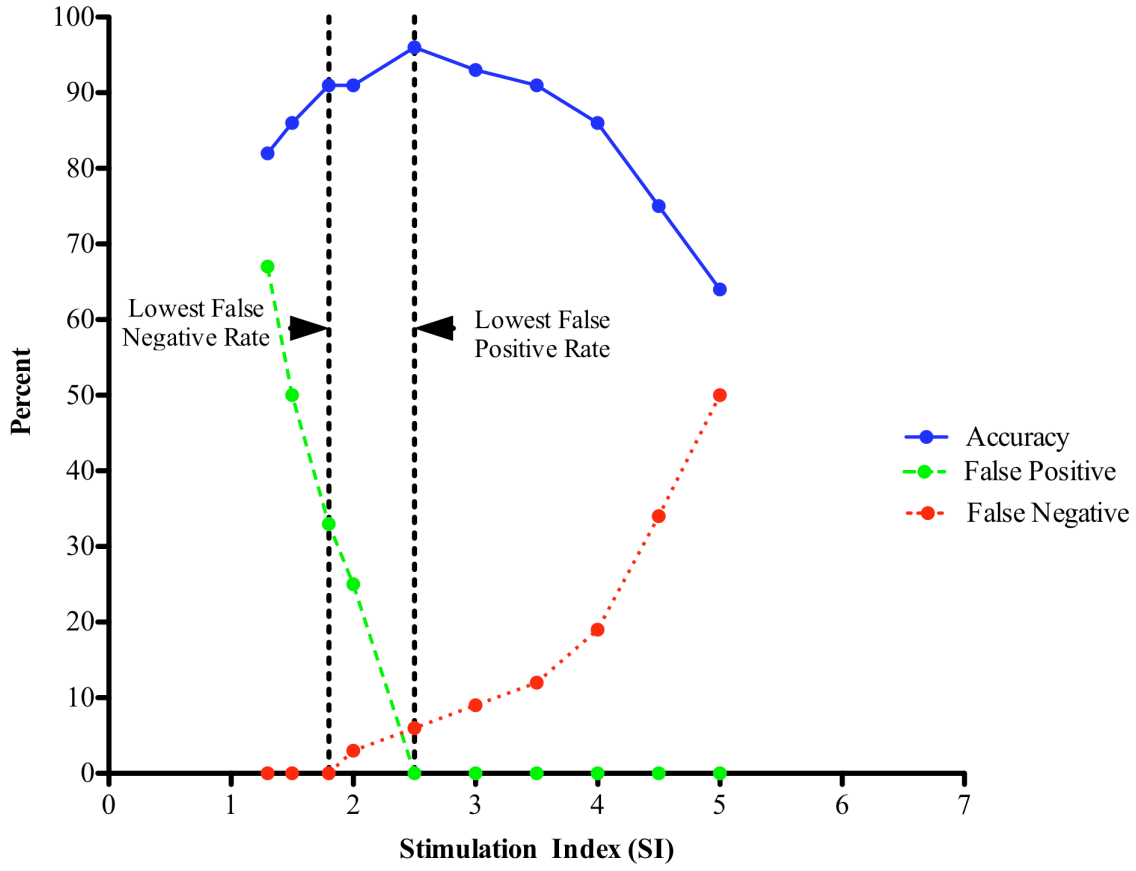
1. The positive/negative outcome for each substance for each criterion was determined by the outcome of the test with the highest maximum stimulation index (SI) of the multiple tests.
2. The positive/negative outcome for each substance for each criterion was determined by the outcome of the test with the lowest maximum SI of the multiple tests.

Section 6.0 of this background review document provides the results for the analysis when the most prevalent outcome was used to represent the result for each substance tested multiple times (for each criterion).

1.1 Results of LLNA: DA Accuracy Analysis Using Alternative Decision Criteria and Highest Maximum SI for the Outcome of Multiple Tests

When combining multiple test results for a single substance by using the outcome of the test with the highest maximum SI to identify potential sensitizers, the decision criterion of SI ≥ 3.0 (used by the LLNA: DA validation study team) yielded an accuracy of 93% (41/44), a sensitivity of 91% (29/32), a specificity of 100% (12/12), a false positive rate of 0% (0/12), and a false negative rate of 9% (3/32) (**Table C-V-1)**. The decision criteria using higher SI values, SI ≥ 3.5 to SI ≥ 5.0, decreased performance except for the specificity and the false positive rate, which remained at 100% (12/12) and 0% (0/12), respectively (**Figure C-V-1** and **Table C-V-1)**. The lower SI criterion, SI ≥ 1.8, decreased accuracy to 91% (40/44) but increased sensitivity to 100% (32/32), while the specificity and the false positive rate decreased to 67% (8/12) and 33% (4/12), respectively. Further, the false negative rate decreased to 0% (0/32) at SI ≥ 1.8. The use of analysis of variance (ANOVA) and summary statistics (i.e., mean ATP measurement of treated groups ≥95% confidence interval [CI] of the control group, or ≥2 or ≥3 standard deviation [SD] from the control group mean), yielded accuracy values of 75% to 84%, with sensitivity values of 88% to 100%, and false negative rates of 0 to 13%. The specificity for these criteria ranged from 8% to 58% and the false positive rates were 42% to 92%. As summarized above, the best overall performance of these alternative decision criteria (based on the highest SI cutoff that yielded no false positives) was achieved using an SI ≥ 1.8 and using the highest maximum SI for substances with more than one test. Using a cutoff at SI ≥ 1.8, however, misclassified four nonsensitizers in the traditional LLNA (including isopropanol based on its highest maximum SI of 1.97).

Figure C-V-1 Performance of the LLNA: DA for 44 Substances Compared to the Traditional LLNA in Predicting Skin Sensitization Potential Using the Highest Maximum SI for Substances with Multiple Tests



As compared to traditional LLNA results, the lines show the change in performance characteristics for the LLNA: DA with the SI cutoff used to identify sensitizers. This analysis used LLNA: DA and traditional LLNA results for 44 substances (32 traditional LLNA sensitizers and 12 traditional LLNA nonsensitizers). For the 14 substances with multiple test results, the result for each substance was based on the test with the highest maximum SI value. The solid line shows accuracy, the dashed line shows the false positive rate, and the dotted line shows the false negative rate.

Abbreviations: LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; SI = stimulation index.

Table C-V-1 Performance of the LLNA: DA for 44 Substances Compared to the Traditional LLNA in Predicting Skin Sensitization Potential Using Alternative Decision Criteria Based on the Highest Maximum SI for Substances with Multiple Tests

Alternate Criterion	N[1]	Accuracy % (No.[2])	Sensitivity % (No.[2])	Specificity % (No.[2])	False Positive Rate % (No.[2])	False Negative Rate % (No.[2])	Positive Predictivity % (No.[2])	Negative Predictivity % (No.[2])
Statistics[3]	44	84 (37/44)	94 (30/32)	58 (7/12)	42 (5/12)	6 (2/32)	86 (30/35)	78 (7/9)
≥95% CI[4]	44	75 (33/44)	100 (32/32)	8 (1/12)	92 (11/12)	0 (0/32)	74 (32/43)	100 (1/1)
≥2 SD[5]	44	77 (34/44)	91 (29/32)	42 (5/12)	58 (7/12)	9 (3/32)	81 (29/36)	63 (5/8)
≥3 SD[6]	44	77 (34/44)	88 (28/32)	50 (6/12)	50 (6/12)	13 (4/32)	82 (28/34)	60 (6/10)
SI ≥ 5.0	44	64 (28/44)	50 (16/32)	100 (12/12)	0 (0/12)	50 (16/32)	100 (16/16)	43 (12/28)
SI ≥ 4.5	44	75 (33/44)	66 (21/32)	100 (12/12)	0 (0/12)	34 (11/32)	100 (21/21)	52 (12/23)
SI ≥ 4.0	44	86 (38/44)	81 (26/32)	100 (12/12)	0 (0/12)	19 (6/32)	100 (26/26)	67 (12/18)
SI ≥ 3.5	44	91 (40/44)	88 (28/32)	100 (12/12)	0 (0/12)	13 (4/32)	100 (28/28)	75 (12/16)
SI ≥ 3.0	*44*	*93 (41/44)*	*91 (29/32)*	*100 (12/12)*	*0 (0/12)*	*9 (3/32)*	*100 (29/29)*	*80 (12/15)*
SI ≥ 2.5	*44*	*96 (42/44)*	*94 (30/32)*	*100 (12/12)*	*0 (0/12)*	*6 (2/32)*	*100 (30/30)*	*86 (12/14)*
SI ≥ 2.0	44	91 (40/44)	97 (31/32)	75 (9/12)	25 (3/12)	3 (1/32)	91 (31/34)	90 (9/10)
SI ≥ 1.8	**44**	**91 (40/44)**	**100 (32/32)**	**67 (8/12)**	**33 (4/12)**	**0 (0/32)**	**89 (32/36)**	**100 (8/8)**
SI ≥ 1.5	44	86 (38/44)	100 (32/32)	50 (6/12)	50 (6/12)	0 (0/32)	84 (32/38)	100 (6/6)
SI ≥ 1.3	44	82 (36/44)	100 (32/32)	33 (4/12)	67 (8/12)	0 (0/32)	80 (32/40)	100 (4/4)

Italicized text indicates the decision criterion chosen by the LLNA: DA validation study team; boldface indicates the single decision criterion that had an overall increased performance in predicting skin sensitization potential when compared to the traditional LLNA (i.e., no false negatives).

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP Content; No. = number; SD = standard deviation; SI = stimulation index

[1] N = Number of substances included in this analysis.

[2] The proportion on which the percentage calculation is based.

[3] Analysis of variance for difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The ATP data were log-transformed prior to statistical analyses. For analysis of variance, significance at $p < 0.05$ was further tested by Dunnett's test.

[4] The mean ATP of at least one treatment group was outside the 95% CI for the mean ATP of the vehicle control group.

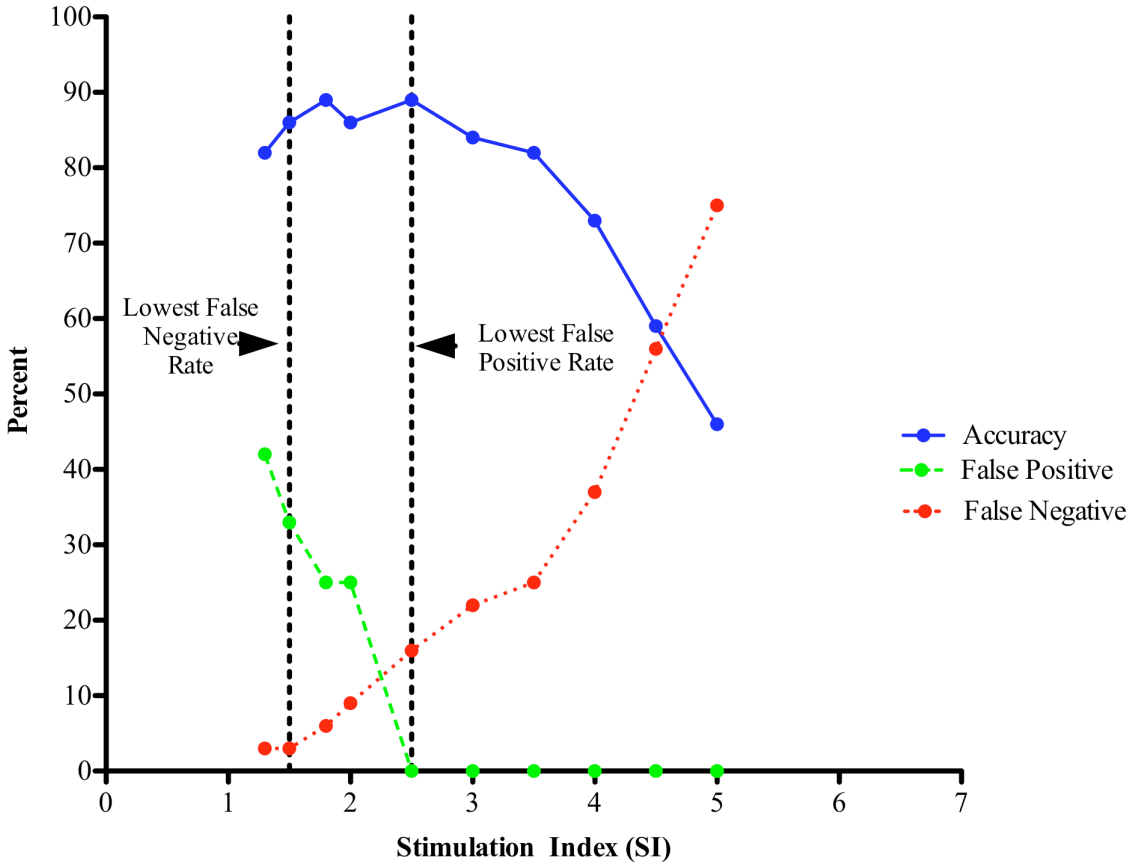
[5] The mean ATP of at least one treatment group was greater than 2 SD from the mean ATP of the vehicle control group.

[6] The mean ATP of at least one treatment group was greater than 3 SD from the mean ATP of the vehicle control group.

1.2 Results of LLNA: DA Accuracy Analysis Using Alternative Decision Criteria and Lowest Maximum SI for the Outcome of Multiple Tests

When combining multiple test results for a single substance using the outcome of the test with the lowest maximum SI to identify potential sensitizers, the decision criterion of SI ≥ 3.0 (used by the LLNA: DA validation study team) yielded an accuracy of 84% (37/44), a sensitivity of 78% (25/32), a specificity of 100% (12/12), a false positive rate of 0% (0/12), and a false negative rate of 22% (7/32) (**Table C-V-2)**. The decision criteria using higher SI values, SI ≥ 3.5 to SI ≥ 5.0, decreased performance except for the specificity and the false positive rate, which remained at 100% (12/12) and 0% (0/12), respectively (**Figure C-V-2** and **Table C-V-2**). At SI ≥ 5.0, accuracy decreased to 46% (20/44) and the false negative rate increased to 75% (24/32). Use of a lower SI cutoff at SI ≥ 2.5 increased accuracy to 89% (39/44) and sensitivity to 84% (27/32), while the specificity and false positive rate remained the same at 100% (12/12) and 0% (0/12), respectively. Further, the false negative rate decreased to 16% (5/32) at SI ≥ 2.5. At SI ≥ 1.8, accuracy was unchanged at 89% (39/44) with an increased sensitivity of 94% (30/32) and decreased false negative rate of 6% (2/32), while specificity was 75% (9/12) and the false positive rate was 25% (3/12). At an even lower SI criterion, SI ≥ 1.3, accuracy was decreased to 86% (38/44) but the sensitivity increased to 97% (31/32), while the specificity was 58% (7/12) and the false positive rate was 42% (5/12). Further, the false negative rate decreased to 3% (1/32) at SI ≥ 1.3. Use of a statistical test (i.e., ANOVA or *t*-test) and summary statistics (i.e., mean ATP measurements of treated groups ≥95% CI of the control group, or ≥2 or ≥3 SD from the control group mean), yielded accuracy values of 77 to 82%, with sensitivity values of 84 to 97%, and false negative rates of 3 to 16%. Both the specificity and false positive rate for these criteria ranged from 42 to 58%. Of these alternative decision criteria, the best overall performance (i.e., lowest combined false positive and false negative rate) for the approach using the lowest maximum SI for substances with more than one test was achieved using SI ≥ 1.8, as summarized above.

Figure C-V-2 Performance of the LLNA: DA for 44 Substances Compared to the Traditional LLNA in Predicting Skin Sensitization Potential Using the Lowest Maximum SI for Substances with Multiple Tests



As compared to traditional LLNA results, the lines show the change in performance characteristics for the LLNA: DA with the SI cutoff used to identify sensitizers. This analysis used LLNA: DA and traditional LLNA results for 44 substances (32 traditional LLNA sensitizers and 12 traditional LLNA nonsensitizers). For the 14 substances with multiple test results, the result for each substance was based on the test with the lowest maximum SI value. The solid line shows accuracy, the dashed line shows the false positive rate, and the dotted line shows the false negative rate.

Abbreviations: LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; SI = stimulation index.

Table C-V-2 Performance of the LLNA: DA for 44 Substances Compared to the Traditional LLNA in Predicting Skin Sensitization Potential Using Alternative Decision Criteria Based on the Lowest Maximum SI for Substances with Multiple Tests

Alternate Criterion	N[1]	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate		Positive Predictivity		Negative Predictivity	
		%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]
Statistics[3]	44	82	36/44	91	29/32	58	7/12	42	5/12	9	3/32	85	29/34	70	7/10
≥95% CI[4]	44	82	36/44	97	31/32	42	5/12	58	7/12	3	1/32	82	31/38	83	5/6
≥2 SD[5]	44	77	34/44	88	28/32	50	6/12	50	6/12	13	4/32	82	28/34	60	6/10
≥3 SD[6]	44	77	34/44	84	27/32	58	7/12	42	5/12	16	5/32	84	27/32	58	7/12
SI ≥ 5.0	44	46	20/44	25	8/32	100	12/12	0	0/12	75	24/32	100	8/8	33	12/36
SI ≥ 4.5	44	59	26/44	44	14/32	100	12/12	0	0/12	56	18/32	100	14/14	40	12/30
SI ≥ 4.0	44	73	32/44	63	20/32	100	12/12	0	0/12	38	12/32	100	20/20	50	12/24
SI ≥ 3.5	44	82	36/44	75	24/32	100	12/12	0	0/12	25	8/32	100	24/24	60	12/20
SI ≥ 3.0	*44*	*84*	*37/44*	*78*	*25/32*	*100*	*12/12*	*0*	*0/12*	*22*	*7/32*	*100*	*25/25*	*63*	*12/19*
SI ≥ 2.5	*44*	*89*	*39/44*	*84*	*27/32*	*100*	*12/12*	*0*	*0/12*	*16*	*5/32*	*100*	*27/27*	*71*	*12/17*
SI ≥ 2.0	44	86	38/44	91	29/32	75	9/12	25	3/12	9	3/32	91	29/32	75	9/12
SI ≥ 1.8	**44**	**89**	**39/44**	**94**	**30/32**	**75**	**9/12**	**25**	**3/12**	**6**	**2/32**	**91**	**30/33**	**82**	**9/11**
SI ≥ 1.5	44	89	39/44	97	31/32	67	8/12	33	4/12	3	1/32	89	31/35	89	8/9
SI ≥ 1.3	44	86	38/44	97	31/32	58	7/12	42	5/12	3	1/32	86	31/36	88	7/8

Italicized text indicates the decision criterion chosen by the LLNA: DA validation study team; boldface indicates the single decision criterion that had an overall increased performance in predicting skin sensitization potential when compared to the traditional LLNA (i.e., lowest combined false positive and false negative rate).

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP Content; No. = number; SD = standard deviation; SI = stimulation index.

[1] N = Number of substances included in this analysis.

[2] The proportion on which the percentage calculation is based.

[3] Analysis of variance for difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The ATP data were log-transformed prior to statistical analyses. For analysis of variance, significance at $p < 0.05$ was further tested by Dunnett's test.

[4] The mean ATP of at least one treatment group was outside the 95% confidence interval for the mean ATP of the vehicle control group.

[5] The mean ATP of at least one treatment group was greater than 2 SD from the mean ATP of the vehicle control group.

[6] The mean ATP of at least one treatment group was greater than 3 SD from the mean ATP of the vehicle control group.

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2.0 Discordant Results for Accuracy Analyses Using Alternative Decision Criteria

This section discusses the discordant results obtained for the analyses using the alternative decision criteria shown in **Tables C-V-1** and **C-V-2**. Discordant results using alternative decision criteria and the highest maximum SI outcome for multiple tests are discussed first (**Section 2.1**), followed by discussion of discordant results using alternative decision criteria and lowest maximum SI outcome for multiple tests (**Section 2.2**). In all cases, discordant results for the alternative decision criteria are discussed using the traditional LLNA as the reference test.

2.1 Discordant Results Using Alternative Decision Criteria and Highest Maximum SI Outcome for Multiple Tests

Table C-V-3 shows how the number and identity of discordant substances changes with the alternative decision criteria when using the test with the highest maximum SI to represent the outcome for substances with multiple tests. Using the decision criterion of SI ≥ 3.0 to identify sensitizers and the test with the highest maximum SI as the representative result for substances with multiple tests yielded three discordant substances (i.e., 3-aminophenol, 2-mercaptobenzothiazole, and methyl methacrylate), all misclassified as nonsensitizers by the LLNA: DA. Using an SI cutoff lower than three to identify sensitizers, such as SI ≥ 2.0, yielded four discordant substances: chlorobenzene, hexane, and salicylic acid were misclassified as sensitizers and methyl methacrylate was misclassified as a nonsensitizer. As mentioned in **Section 1.1**, using the decision criterion of SI ≥ 1.8 to identify sensitizers (based on the test with the highest maximum SI for substances with multiple test results) yielded the highest SI cutoff with no false negatives among the alternative decision criteria evaluated. Yet, when compared to the traditional LLNA, four substances (chlorobenzene, hexane, isopropanol, and salicylic acid) were misclassified as sensitizers by the LLNA: DA. Using a lower SI cutoff of SI ≥ 1.3 to identify sensitizers, yielded eight discordant substances that were all misclassified as sensitizers (i.e., 1-bromobutane, dimethyl isophthalate, methyl salicylate, and nickel [II] chloride plus the four substances misclassified at SI ≥ 1.8). Increasing the SI cutoff to values greater than three increased the number of sensitizers that were misclassified as nonsensitizers. At SI ≥ 4.0, six traditional LLNA sensitizers were misclassified as nonsensitizers by the LLNA: DA while at SI ≥ 5.0, 16 sensitizers were misclassified as nonsensitizers (**Table C-V-3)**.

Use of a statistical test (i.e., ANOVA or *t*-test) or summary statistics (i.e., ≥95% CI, ≥2 SD, or ≥3 SD) tended to misclassify nonsensitizers in the traditional LLNA as sensitizers in the LLNA: DA. Using ANOVA or *t*-test to identify sensitizers misclassified five nonsensitizers (i.e., 1-bromobutane, chlorobenzene, hexane, salicylic acid, and sulfanilamide) as sensitizers and two sensitizers (i.e., 2-mercaptobenzothiazole and methyl methacrylate) as nonsensitizers. Using treatment group ATP measurement with ≥2 SD or ≥3 SD of the vehicle control mean or a ≥95% CI of the vehicle control mean, all misclassified the following six traditional LLNA nonsensitizers as sensitizers: 1-bromobutane, chlorobenzene, hexane, isopropanol, nickel (II) chloride, and propylparaben. The ≥95% CI of the vehicle control mean misclassified four additional nonsensitizers (i.e., diethyl phthalate, dimethyl isophthalate, lactic acid, and methyl salicylate) as sensitizers. In addition, ≥2 SD or ≥3 SD of the vehicle control mean commonly misclassified three sensitizers as nonsensitizers (i.e., ethyl acrylate, methyl methacrylate, and propyl gallate).

Thirteen of the 22 ICCVAM-recommended LLNA performance standards reference substances (ICCVAM 2009) tested in the LLNA: DA were discordant for the analysis of alternative decision criteria using the test with the highest maximum SI to represent substances with multiple tests (**Table C-V-3**) when compared to the traditional LLNA. Six nonsensitizers in the traditional LLNA (i.e., chlorobenzene, isopropanol, lactic acid, methyl salicylate, nickel [II] chloride, and salicylic acid) were misclassified by some criteria in the LLNA: DA as a sensitizers, and seven sensitizers in the

traditional LLNA (i.e., citral, ethylene glycol dimethacrylate, imidazolidinyl urea, 2-mercaptobenzothiazole, methyl methacrylate, phenyl benzoate, and sodium lauryl sulfate) were misclassified as nonsensitizers by some criteria when tested in the LLNA: DA.

2.2 Discordant Results Using Alternative Decision Criteria and Lowest Maximum SI Outcome for Multiple Tests

Table C-V-4 shows how the number and identity of discordant substances changes with the alternative decision criteria when using the test with the lowest maximum SI as the representative result for substances with multiple tests. Using an SI cutoff less than three, SI ≥ 2.0, to identify sensitizers yielded six discordant substances. Three of the six discordant substances (i.e., 3-aminophenol, methyl methacrylate, and nickel [II] sulfate hexahydrate) were misclassified as nonsensitizers by the LLNA: DA compared to the traditional LLNA and the remaining three (i.e., chlorobenzene, hexane, and salicylic acid) were misclassified as sensitizers. As mentioned in **Section 1.2**, using the decision criterion of SI ≥ 1.8 to identify sensitizers (based on the test with the lowest maximum SI for substances with multiple tests) yielded optimum performance (i.e., lowest combined false positive and false negative rate) for the LLNA: DA when compared to the traditional LLNA. This decision criterion yielded five discordant substances; two were sensitizers in the traditional LLNA but were misclassified as nonsensitizers in the LLNA: DA (i.e., 3-aminophenol and nickel [II] sulfate hexahydrate) and three were nonsensitizers in the traditional LLNA but were misclassified as sensitizers in the LLNA: DA (i.e., chlorobenzene, hexane, and salicylic acid) (**Table C-V-4**).

Using an even lower SI to identify sensitizers, SI ≥ 1.3, also yielded six discordant substances. Chlorobenzene, hexane, and salicylic acid were still misclassified as sensitizers and nickel (II) sulfate hexahydrate was still misclassified as a nonsensitizer by the LLNA: DA compared to the traditional LLNA. In addition, 1-bromobutane and nickel (II) chloride were also misclassified as sensitizers. Increasing the SI cutoff to values greater than three increased the number of sensitizers that were misclassified as nonsensitizers. At SI ≥ 4.0, 12 sensitizers were misclassified as nonsensitizers while at SI ≥ 5.0, 24 sensitizers were misclassified as nonsensitizers. Using the test with the lowest maximum SI as the result for substances with multiple tests caused even potent sensitizers to be misclassified as nonsensitizers at the higher SI cutoffs. For instance, at SI ≥ 5.0, 2,4-dinitrochlorobenzene and glutaraldehyde were classified as nonsensitizers (**Table C-V-4)**.

Use of a statistical test (i.e., ANOVA or *t*-test) or summary statistics (i.e., ≥95% CI, ≥2 SD, or ≥3 SD) more often misclassified traditional LLNA nonsensitizers than sensitizers (**Table C-V-4**). Using ANOVA or *t*-test to identify sensitizers misclassified three sensitizers in the traditional LLNA (i.e., 2-mercaptobenzothiazole, methyl methacrylate, and nickel [II] sulfate hexahydrate) as nonsensitizers in the LLNA: DA. Further, five nonsensitizers in the traditional LLNA (i.e., 1-bromobutane, chlorobenzene, hexane, salicylic acid, and sulfanilamide) were misclassified as sensitizers in the LLNA: DA. Using treatment group ATP measurement ≥95% CI, ≥2 SD or ≥3 SD of vehicle control mean commonly misclassified 1-bromobutane, chlorobenzene, hexane, nickel (II) chloride, and propylparaben as sensitizers and nickel (II) sulfate hexahydrate as a nonsensitizer compared to traditional LLNA results. In addition each summary statistic misclassified from two to four additional substances when compared to traditional LLNA results (see **Table C-V-4**).

Table C-V-3 Discordant Results for the LLNA: DA Using Alternative Decision Criteria Compared to the Traditional LLNA Based on the Highest Maximum SI for Substances with Multiple Tests

Discordant Substance[1]	Alternative Decision Criterion[2]													
	Statistics[3]	≥95% CI[4]	≥2 SD[5]	≥3 SD[6]	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.8	SI ≥ 1.5	SI ≥ 1.3
3-Aminophenol (3.2%)					-	-	-	-	-					
p-Benzoquinone (0.01%)					-	-	-							
1-Bromobutane (-)	+	+	+	+									+	+
Butyl glycidyl ether (30.9%)				-	-									
Chlorobenzene (-)	+	+	+	+							+	+	+	+
Cinnamic aldehyde (1.9%)					-									
Citral (9.2%)					-	-								
Diethyl maleate (3.6%)					-	-	-							
Diethyl phthalate (-)		+												
Dimethyl isophthalate (-)		+												+
Ethyl acrylate (32.8%)			-	-	-	-								
Ethylene glycol dimethacrylate (28.0%)					-	-								
Hexane (-)	+	+	+	+							+	+	+	+
Imidazolidinyl urea (24.0%)					-									
Isopropanol (-)		+	+	+								+	+	+
Lactic acid (-)		+												
2-Mercaptobenzothiazole (1.7%)	-				-	-	-	-	-	-				

Discordant Substance[1]	Alternative Decision Criterion[2]													
	Statistics[3]	≥95% CI[4]	≥2 SD[5]	≥3 SD[6]	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.8	SI ≥ 1.5	SI ≥ 1.3
Methyl methacrylate (90.0%)	-		-	-	-	-	-	-	-	-	-			
Methyl salicylate (-)		+											+	+
Nickel (II) chloride (-)		+	+	+										+
Phenyl benzoate (13.6%)					-	-								
Propyl gallate (0.32%)			-	-	-									
Propylparaben (-)		+	+	+										
Resorcinol (6.3%)					-	-								
Salicylic acid (-)	+	+	+								+	+	+	+
Sodium lauryl sulfate (8.1%)					-	-	-	-						
Sulfanilamide (-)	+													
Trimellitic anhydride (4.7%)					-									

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP Content; SD = standard deviation; SI = stimulation index.

[1] Compared to the traditional LLNA; traditional LLNA result in parentheses are "-" for nonsensitizers and EC3 values for sensitizers.

[2] LLNA: DA outcomes are indicated by "+" for sensitizer results and "-" for nonsensitizer results.

[3] Analysis of variance assessed difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The ATP data were log-transformed prior to statistical analyses. Significance by analysis of variance at $p < 0.05$ was further tested by Dunnett's test.

[4] The mean ATP of at least one treatment group was outside the 95% CI for the mean ATP of the vehicle control group.

[5] The mean ATP of at least one treatment group was greater than 2 SD from the mean ATP of the vehicle control group.

[6] The mean ATP of at least one treatment group was greater than 3 SD from the mean ATP of the vehicle control group.

Table C-V-4 Discordant Results for the LLNA: DA Using Alternative Decision Criteria Compared to the Traditional LLNA Based on the Lowest Maximum SI for Substances with Multiple Tests

Discordant Substance[1]	Alternative Decision Criterion[2]													
	Statistics[3]	≥95% CI[4]	≥2 SD[5]	≥3 SD[6]	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.8	SI ≥ 1.5	SI ≥ 1.3
Abietic Acid (11.9%)					-	-	-							
3-Aminophenol (3.2%)					-	-	-	-	-	-	-	-		
p-Benzoquinone (0.01%)					-	-	-							
1-Bromobutane (-)	+	+	+	+									+	+
Butyl glycidyl ether (30.9%)				-	-									
Chlorobenzene (-)	+	+	+	+							+	+	+	+
Cinnamic aldehyde (1.9%)					-									
Citral (9.2%)					-	-								
Cobalt chloride (0.60%)					-	-	-	-	-	-				
Diethyl phthalate (-)		+												
Dimethyl isophthalate (-)														
Diethyl maleate (3.6%)					-	-	-							
2,4-Dinitrochlorobenzene (0.05%)					-									
Ethyl acrylate (32.8%)			-	-	-	-								
Ethylene glycol dimethacrylate (28.0)					-	-								
Formaldehyde (0.50%)					-	-	-	-	-					
Glutaraldehyde (0.08%)					-	-	-	-	-					

Discordant Substance[1]	Alternative Decision Criterion[2]													
	Statistics[3]	≥95% CI[4]	≥2 SD[5]	≥3 SD[6]	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.8	SI ≥ 1.5	SI ≥ 1.3
Hexane (-)	+	+	+	+							+	+	+	+
Hexyl cinnamic aldehyde (9.7%)					-	-	-							
Imidazolidinyl urea (24.0%)					-									
2-Mercaptobenzothiazole (1.7%)	-				-	-	-	-	-	-				
Methyl methacrylate (90.0%)	-		-	-	-	-	-	-	-	-	-			
Nickel (II) chloride (-)		+	+	+										+
Nickel (II) sulfate hexahydrate (4.8%)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phenyl benzoate (13.6%)					-	-								
Potassium dichromate (0.17%)					-	-								
Propyl gallate (0.32%)			-	-	-									
Propylparaben (-)		+	+	+										
Resorcinol (6.3%)					-	-								
Salicylic acid (-)	+	+	+								+	+	+	+
Sulfanilamide (-)	+													
Sodium lauryl sulfate (8.1%)					-	-	-	-						
Trimellitic anhydride (4.7%)					-									

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP Content; SD = Standard deviation; SI = Stimulation index.

[1] Compared to the traditional LLNA; traditional LLNA result in parentheses are "-" for nonsensitizers and EC3 values for sensitizers.

[2] LLNA: DA outcomes are indicated by "+" for sensitizer results and "-" for nonsensitizer results.

[3] Analysis of variance for difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The ATP data were log-transformed prior to statistical analyses. Significance by analysis of variance at $p < 0.05$ was further tested by Dunnett's test.

[4] The mean ATP of at least one treatment group was outside the 95% CI for the mean ATP of the vehicle control group.

[5] The mean ATP of at least one treatment group was greater than 2 SD from the mean ATP of the vehicle control group.

[6] The mean ATP of at least one treatment group was greater than 3 SD from the mean ATP of the vehicle control group.

Thirteen of the 22 ICCVAM-recommended LLNA performance standards reference substances (ICCVAM 2009) were discordant for the analysis of alternative decision criteria using the test with the lowest maximum SI as the representative result for substances with multiple tests (**Table C-V-4**). One strong sensitizer in the traditional LLNA, 2,4-dinitrochlorobenzene, was misclassified by SI ≥ 5.0 as a nonsensitizer in the LLNA: DA. Nine additional sensitizers (i.e., citral, cobalt chloride, ethylene glycol dimethacrylate, hexyl cinnamic aldehyde, imidazolidinyl urea, 2-mercaptobenzothiazole, methyl methacrylate, phenyl benzoate, and sodium lauryl sulfate) were also misclassified as nonsensitizers by some criteria in the LLNA: DA. Three nonsensitizers in the traditional LLNA (i.e., chlorobenzene, nickel [II] chloride, and salicylic acid) were misclassified as sensitizers by some criteria in the LLNA: DA.

Annex VI

Evaluation of the Robustness of the SI Cutoff Criteria Used for the LLNA: BrdU-ELISA and LLNA: DA Test Methods

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1.0 Evaluation of the Robustness of the SI Cutoff Criteria Used for the LLNA: BrdU-ELISA and LLNA: DA Test Methods

The analyses described in this annex aim to determine the robustness of the optimum stimulation index (SI) criteria for the murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine (LLNA: BrdU-ELISA) and murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content (LLNA: DA) test methods. The analyses show that the optimal SI criteria for the LLNA: DA and the LLNA: BrdU-ELISA test methods are quite stable. Taking different samples of the data as training/validation sets has relatively little impact on the cutoff SI criteria or on the resulting number of false positives or false negatives. Both assays perform quite well for the optimized SI cutoff criteria. The proposed SI cutoff criteria should be adopted for now and reoptimized in the future after new prospective data have been collected.

1.1 Basis for Selection of the Optimized Criteria

The optimum SI criteria proposed in Section 6.5 of the background review document (BRD) were based on selecting the highest SI values that produced no false negatives, relative to traditional murine local lymph node assay (LLNA) outcomes, in the entire databases of 43 (LLNA: BrdU-ELISA) or 44 (LLNA: DA) substances. Substances with multiple test results are represented by the most prevalent outcome for the SI criterion evaluated (e.g., if a substance had more negative than positive results at SI $\geq$ 1.6, then the substance was deemed negative). If there were an equal number of positive and negative tests for a substance at a particular SI criterion, then a conservative approach was taken where the substance was deemed positive at that criterion in order to be protective of public health. The "most prevalent outcome" approach is the same as using the median SI, or the higher of the two SI values in the middle of the data if there are an equal number of SI values.

1.2 Methods

Since there were no newly tested substances for which the optimized cutoff criteria (currently proposed to be SI $\geq$ 1.6 for the LLNA: BrdU-ELISA test method and SI $\geq$ 1.8 for the LLNA: DA test method) could be prospectively applied, a retrospective evaluation was performed. This retrospective validation involved taking various samples of the existing data as training sets, reoptimizing the SI cutoff criteria, and then applying the new criteria to the remainder of the data, which would serve as a validation set.

Such a validation exercise can be useful for situations in which the decision criteria for distinguishing between "positives" and "negatives" are quite complex and involve multiple variables. In such cases, it is quite common to discover that an apparently "successful" decision criteria based on a training set is really just an artifact unique to those substances, and cannot be generalized or extrapolated to another set of substances, such as a validation set. However, the LLNA: BrdU-ELISA and LLNA: DA criteria are extremely simple – a single SI cutoff value, which nevertheless produces an outstanding performance: no false negatives and only two false positives (<5%) for 43 LLNA: BrdU-ELISA-tested substances, and no false negatives and only three false positives (<7%) for the 44 LLNA: DA-tested substances. This excellent performance for a single SI cutoff criterion strongly argues that the criterion is robust to sampling.

When carrying out a validation exercise for the LLNA: BrdU-ELISA and LLNA: DA data, it is important to understand that only a small number of substances actually contribute to the determination and stability of the SI cutoff criterion. Thus, rather than taking various samples of the total dataset, one possible approach is a complete enumeration of all possible samples as it relates to the critical substances. Thus, one validation exercise carried out for the LLNA: BrdU-ELISA and LLNA: DA datasets was to look at all possible sample combinations of the four critical substances and examine the robustness of the optimized cutoff criterion in each case. In addition, a more

traditional validation exercise for both the LLNA: BrdU-ELISA and LLNA: DA datasets was performed. The datasets were first divided into phase I and phase II groups based on the dates that the data were submitted to NICEATM. The phase I substances were considered to be the training set and the phase II substances were considered to be the validation set (and vice versa).

1.3 LLNA: BrdU-ELISA Results

The LLNA: BrdU-ELISA data for 43 substances are summarized and organized by test phase in **Table C-VI-1**. The decision rule applied to the data and the corresponding SI cutoff point were designed to minimize false positives while eliminating false negatives. As indicated above, the results were impressive, with a very low (<5%) false positive rate when using SI ≥ 1.6 as the cutoff point.

It was noted that choosing SI ≥ 1.5 would produce exactly the same result as SI ≥ 1.6 for the 43 LLNA: BrdU-ELISA substances (no false negatives; two false positives). Choosing the lower critical value of 1.5 would minimize the likelihood of a false negative in the testing of future substances, while SI ≥ 1.6 minimizes the likelihood of future false positives. The calculations that follow use SI ≥ 1.6 as the critical cutoff. This same issue arises for the LLNA: DA data (see **Section 1.4** of this annex**)**. The SI ≥ 1.6 criterion was selected for the LLNA: BrdU-ELISA database because it was the highest SI value that produced no false negatives with minimal false positives.

For the first analysis, half of the LLNA: BrdU-ELISA substances were sampled to form a training set, while the remainder of the data served as the validation set. For each sample, the SI cutoff was re-optimized using the substances in the training set and then applied to the validation set. Because the criterion must be optimized to prevent false negatives and minimize the number of false positives, the SI cutoff is determined solely by the smallest positive SI response of the true positive substances in the training set. Thus in a sample, the cutoff SI can only increase, never decrease, relative to the cutoff SI for entire database. Similarly, the false positive rate in the validation set can only go down, while the false negative rate can and does go up based on the cutoff value selected using the training set.

Table C-VI-1 SI Data for the LLNA: BrdU-ELISA[1]

Substance Name	SI for True Positives[2]	Substance Name	SI for True Negatives[3]
Phase I (N = 31)			
Citral	16.35	Hexane	1.89
1, 4-Phenylenediamine	14.70	Lactic acid	1.89
Glutaraldehyde	14.60	Methyl salicylate	1.43
Diphenylcyclopropenone	11.62	Glycerol	1.29
Trimellitic anhydride	7.85	Dimethyl isophthalate	1.26
p-Benzoquinone	6.90	Propylene glycol	1.20
2, 4-Dinitrochlorobenzene	6.84	2-Hydroxypropyl-methacrylate	1.13
Isoeugenol	6.73	Isopropanol	1.01
Cyclamen aldehyde	5.71	Diethyl phthalate	0.88
Hydroxycitronellal	4.78		
Linalool	4.65		
Formaldehyde	4.40		

continued

Table C-VI-1 SI Data for the LLNA: BrdU-ELISA[1] (continued)

Substance Name	SI for True Positives[2]	Substance Name	SI for True Negatives[3]
Phase I (N = 31)			
Isopropyl myristate	4.19		
Cinnamic aldehyde	3.97		
trans-Cinnamaldehyde	3.50		
Hexyl cinnamic aldehyde	3.40		
Eugenol	3.30		
3-Aminophenol	3.06		
Nickel sulfate	2.66		
4-Chloroaniline	2.53		
Aniline	2.07		
2-Mercaptobenzothiazole	1.62		
Phase II (N = 12)			
Diethyl maleate	6.27	Salicylic acid	1.26
Ethyl acrylate	4.95	Sulfanilamide	1.26
5-Chloro-2-methyl-4-isothiazolin-3-one solution	4.83		
4-Methylaminophenol sulfate	3.98		
Cobalt chloride	3.68		
Phenyl benzoate	3.37		
Ethylene glycol dimethacrylate	3.11		
Cinnamic alcohol	2.74		
Sodium lauryl sulfate	2.64		
Imidazolidinyl urea	1.61		

Abbreviations: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; N = number of substances; SI = stimulation index.

[1] Substances with multiple test results are represented by the median SI, or the highest of the two SI values in the middle of the data if there are an equal number of SI values.

[2] True positives are substances that are positive in the traditional LLNA.

[3] True negatives are substances that are negative in the traditional LLNA.

The most critical substances for the LLNA: BrdU-ELISA data when evaluating the stability of the cutoff SI are the four lowest SI values for traditional LLNA positive substances. All of the 16 possible combinations of these substances are provided in **Table C-VI-2**.

Table C-VI-2 All Possible Distributions of Four Key Substances in Training (T) or Validation (V) Sets for LLNA: BrdU-ELISA

4-Chloro-aniline (SI = 2.53)	Aniline (SI = 2.07)	2-Mercapto-benzothiazole (SI = 1.62)	Imidizolidinyl urea (SI = 1.61)	Cutoff SI[1]	Validation Set	
					No. False Positives[2]	No. False Negatives
T	T	T	T	1.6	0-2	0
T	T	T	V	1.6	0-2	0
T	T	V	T	1.6	0-2	0
T	T	V	V	2.0	0	2
T	V	T	T	1.6	0-2	0
T	V	T	V	1.6	0-2	0
T	V	V	T	1.6	0-2	0
T	V	V	V	2.5	0	3
V	T	T	T	1.6	0-2	0
V	T	T	V	1.6	0-2	0
V	T	V	T	1.6	0-2	0
V	T	V	V	2.0	0	2
V	V	T	T	1.6	0-2	0
V	V	T	V	1.6	0-2	0
V	V	V	T	1.6	0-2	0
V	V	V	V	>2.5	0	≥4

Abbreviations: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; No. = number; SI = stimulation index; T = substance was in the training set; V = substance was in the validation set.

[1] The cutoff value is determined using the training set.

[2] The number of false positives in the validation set depend upon whether the two LLNA: BrdU-ELISA false positives with SI > 1.6, lactic acid (SI = 1.89) and hexane (SI = 1.89), are in the training set or in the validation set.

The cutoff SI values are relatively stable for the LLNA: BrdU-ELISA. The likelihood is 75% (12/16) that a validation exercise would result in an unchanged cutoff of SI ≥ 1.6, which also was the case when the phase I substances were used as the training set and the phase II substances were used as the validation set (and vice versa). The likelihood is 12.5% (2/16) that the cutoff will be elevated to SI ≥ 2, 6.25% (1/16) that it will be elevated to SI ≥ 2.5, and also 6.25% (1/16) that the reoptimized cutoff SI will exceed 2.5. The higher the cutoff SI, the greater the number of false negatives, as can be seen from **Table C-VI-2**. It is also important to recognize that most of the data are not relevant to determining the cutoff SI point. Only the "weakest positives" are critical, and the greater the variability among the SI values for these critical substances, the less stable the cutoff SI points will be.

The second validation exercise considered the phase I substances as a training set and the phase II substances as a validation set (and vice versa). If the phase I data are used as the training set, the SI cutoff point remains unchanged at ≥1.6; if the phase II data are used as the training set, then the SI cutoff point also remains unchanged (≥1.6). If the phase I data cutoff point was used in the evaluation

of phase II substances, then there would be no false positives or false negatives. Conversely, if the phase II cutoff point was used to evaluate the substances in phase I, then there would be no false negatives and two false positives. Once again, the results of the validation study produce quite stable results.

1.4 LLNA: DA Results

The LLNA: DA data for 44 substances are organized by test phase and summarized in **Table C-VI-3**. Again, the decision rule applied to the data and the corresponding SI cutoff point were designed to minimize false positives while totally eliminating false negatives. These data showed a low (<7%) false positive rate. The cutoff value was set at SI ≥ 1.8 based on the data from the 44 substances, although a lower cutoff point, namely SI ≥ 1.7, would have performed exactly the same for these 44 substances (no false negatives; three false positives).

For the first analysis, half of the LLNA: DA substances were sampled to form a training set, while the remainder of the data served as a validation set. For each sample, the SI cutoff is reoptimized based on the substances in the training set and then applied to the validation set. Because the criterion must be optimized to prevent false negatives and minimize the number of false positives, the SI cutoff is determined solely by the smallest SI responses of the true positive substances in the training set. Thus in a sample, the cutoff SI can only increase, never decrease, relative to the cutoff SI for entire database. Similarly, the false positive rate in the validation set can only go down, while the false negative rate can and does go up based on the cutoff value selected using the training set.

Table C-VI-3 SI Data for the LLNA: DA[1]

Substance Name	SI for True Positives[2]	Substance Name	SI for True Negatives[3]
Phase I (N = 31)			
2, 4-Dinitrochloro-benzene	9.96	Chlorobenzene	2.44
Isoeugenol	7.09	Hexane	2.31
Eugenol	7.07	1-Bromobutane	1.65
Benzalkonium chloride	6.68	Methyl salicylate	1.55
Abietic acid	6.26	Propylparaben	1.28
Hydroxycitronellal	5.69	Dimethyl isophthalate	1.26
Hexyl cinnamic aldehyde	5.50	Isopropanol	1.21
Phthalic anhydride	5.49	Diethyl phthalate	1.09
Potassium dichromate	5.49	Lactic acid	0.97
p-Phenylenediamine	5.14		
Glutaraldehyde	5.00		
Trimellitic anhydride	4.96		
Formaldehyde	4.84		
Cinnamic aldehyde	4.73		
Imidazolidinyl urea	4.67		
Citral	4.40		
Resorcinol	4.33		
Cobalt chloride	4.25		

continued

Table C-VI-3 SI Data for the LLNA: DA[1] (continued)

Substance Name	SI for True Positives[1]	Substance Name	SI for True Negatives[2]
Phase I (N = 31)			
Sodium lauryl sulfate	3.39		
3-Aminophenol	2.38		
Nickel (II) sulfate hexahydrate	2.13		
2-Mercaptobenzothiazole	2.00		
Phase II (N = 13)			
5-Chloro-2-methyl-4-isothiazolin-3-one	7.50	Salicylic acid	2.00
Cinnamic alcohol	5.66	Nickel (II) chloride	1.30
Propyl gallate	4.95	Sulfanilamide	0.86
Butyl glycidyl ether	4.59		
Ethylene glycol dimethacrylate	4.45		
Ethyl acrylate	4.29		
Phenyl benzoate	4.24		
p-Benzoquinone	3.79		
Diethyl maleate	3.78		
Methyl methacrylate	1.81		

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; N = number of substances; SI = stimulation index.

[1] Substances with multiple test results are represented by the median SI, or the highest of the two SI values in the middle of the data if there are an equal number of SI values.

[2] True positives are substances that are positive in the traditional LLNA.

[3] True negatives are substances that are negative in the traditional LLNA.

The four most critical substances for the LLNA: DA data when evaluating the stability of the cutoff SI are the four lowest SI values for positive substances. All of the 16 possible combinations of these substances are given in **Table C-VI-4**.

Table C-VI-4 All Possible Distributions of Four Key Substances in Training (T) or Validation (V) Sets for LLNA: DA

3-Aminophenol (SI = 2.38)	Nickel sulfate (SI = 2.13)	2-Mercapto-benzothiazole (SI = 2.00)	Methyl methacrylate (SI = 1.81)	Cutoff SI[1]	Validation Set	
					No. False Positives[2]	No. False Negatives
T	T	T	T	1.8	0-3	0
T	T	T	V	2.0	0-3	1
T	T	V	T	1.8	0-3	0
T	T	V	V	2.1	0-2	2
T	V	T	T	1.8	0-3	0
T	V	T	V	2.0	0-3	1
T	V	V	T	1.8	0-3	0
T	V	V	V	2.3	0-2	3
V	T	T	T	1.8	0-3	0
V	T	T	V	2.0	0-3	1
V	T	V	T	1.8	0-3	0
V	T	V	V	2.1	0-2	2
V	V	T	T	1.8	0-3	0
V	V	T	V	2.0	0-3	1
V	V	V	T	1.8	0-3	0
V	V	V	V	>2.3	0-2	≥4

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; No. = number; SI = stimulation index; T = substance was in the training set; V = substance was in the validation set.

[1] The cutoff value is determined using the training set.

[2] The number of false positives in the validation set depends upon whether the three LLNA: DA false positives (salicylic acid [SI = 2.0], hexane [SI = 2.31], and chlorobenzene [SI = 2.44]) are in the training set or in the validation set.

The cutoff SI values are relatively robust for the LLNA: DA test method also. The likelihood is 50% (8/16) that a validation exercise would result in an unchanged cutoff of SI ≥ 1.8. The likelihood is 25% (4/16) that the cutoff will be increased slightly to SI ≥ 2.0. The likelihood is 12.5% (2/16) that the cutoff will be elevated to SI ≥ 2.1, 6.25% (1/16) that it will be SI ≥ 2.3, and 6.25% (1/16) that it will be greater than 2.3.

This conclusion regarding the stability of the cutoff SI is supported by the phase I vs. phase II approach to validation. This approach considered the phase I substances as a training set and the phase II substances as a validation set (and vice versa). If the phase I LLNA: DA data are used as the training set, the optimized cutoff SI criterion increases slightly from 1.8 to 2.0. If the phase II data are used as the training set, then the SI cutoff criterion remains unchanged at ≥1.8. If the phase I data cutoff point was used in the evaluation of phase II substances, then there would be one false positive and one false negative (methyl methacrylate, SI ≥ 1.81). Conversely, if the phase II cutoff point was used to evaluate the substances in phase I, then there would be no false negatives and two false positives.

1.5 Conclusions

These analyses show that the SI criteria for the LLNA: DA and LLNA: BrdU-ELISA test methods are quite robust. Taking different samples of the data as training/validation sets has relatively little impact on cutoff SI criteria or on the number of false positives or false negatives. Both assays perform quite well for the optimized SI cutoff criteria. The proposed SI cutoff criteria should be adopted for now, and reoptimized in the future after new prospective data have been collected.

Annex VII

Analyses Using Multiple SI Decision Criteria

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1.0 Introduction

This annex provides analyses associated with using two decision criteria for classifying substances using the results from the murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content (LLNA: DA); one criterion to classify substances as sensitizers and another criterion to classify substances as nonsensitizers. The data used for the analyses in this annex are the LLNA: DA results for the 44 substances (32 traditional murine local lymph node assay [LLNA] sensitizers and 12 traditional LLNA nonsensitizers) that were reviewed by the independent peer review panel at the public meeting on April 28-29, 2009. **Section 2** of this annex discusses the accuracy produced by using the two decision criteria and includes an evaluation of discordant, or indeterminate, substances that produced stimulation index (SI) values between the sensitizer and nonsensitizer SI criteria. **Section 3** provides the reproducibility analysis using the decision criterion for sensitizers (**Sections 3.1** and **3.2**) and for tests yielding SI values in three categories: sensitizer, nonsensitizer, and indeterminate (i.e., in the range of uncertainty) (**Section 3.3**). The two SI values determined to be optimal were based on four animals per dose group, and resulted in nine substances that could not be definitively classified because they produced SI values in the range of uncertainty. **Section 4** describes the impact of sample size on the range of the uncertainty between the sensitizer and nonsensitizer criteria. **Section 5** evaluates a number of physicochemical characteristics and other parameters to distinguish between traditional LLNA sensitizers and nonsensitizers in the LLNA: DA, when using multiple SI decision criteria, for potential use in providing additional information for classifying substances that produce SI values in the range of uncertainty.

2.0 Accuracy Analysis Using Multiple Stimulation Index Decision Criteria

As detailed in Section 6.5 of the background review document (BRD), the accuracy of the LLNA: DA when using various single alternative decision criteria was evaluated using the traditional LLNA as the reference test. Compared to the traditional LLNA (SI $\geq$ 3.0), the optimum performance (accuracy of 93% [41/44] and sensitivity of 100% [32/32]) was achieved using the decision criterion of SI $\geq$ 1.8 (Table C-8 of the BRD). Although the SI $\geq$ 1.8 produced a false positive rate of 25% (3/12) it yielded a false negative rate of 0% (0/32) (Table C-8 of the BRD). Increasing the SI decision criterion to SI $\geq$ 2.5 decreased the false positive rate to 0% (0/12) but increased the false negative rate to 13% (4/32). The 0% false positive rate using SI $\geq$ 2.5 and the 0% false negative rate using SI $\geq$ 1.8 prompted an evaluation using two SI decision criteria for determining LLNA: DA results: one criterion to classify substances as sensitizers (SI $\geq$ 2.5) and one criterion to classify substances as nonsensitizers (SI $\leq$ 1.8). The evaluation of this accuracy analysis is described below.

It should be noted that this analysis was based on the same strategy for combining results as that described in Section 6.5 of the BRD for the substances tested multiple times (i.e., the sensitizer/nonsensitizer outcome for each substance using the most prevalent outcome). **Section 3.0** details the reproducibility of substances tested multiple times and indicates that, there were no instances of false positive results for nonsensitizers (0% [0/80] of the substances classified as traditional LLNA nonsensitizers had an SI $\geq$ 2.5 in the LLNA: DA). See **Section 3.0** for more details regarding these results.

2.1 Indeterminate Results Using Multiple Stimulation Index Decision Criteria

While optimum false positive and false negative rates can be achieved for the 44 substances evaluated in the LLNA: DA accuracy analyses using these two different decision criteria, a range of SI values (i.e., between 1.8 and 2.5) exists for which the correct classification is not definitive (i.e., there is a chance for false positive or false negative results for substances that produce SI values in this range). Chemical class, physical form, molecular weight, peptide reactivity (see Annex II of the BRD for physicochemical properties), traditional LLNA EC3 range (estimated concentration needed to

produce a stimulation index of 3) (Table C-2 of the BRD), or potential for skin irritation (Annex III of the BRD) were examined to identify commonalities among the substances that produced SI values between 1.8 and 2.5 in an attempt to identify similar characteristics among these substances that could be used to correctly classify such substances. **Section 5.0** of this annex provides a comprehensive evaluation of a number of physicochemical characteristics and other parameters using the LLNA: DA database to distinguish between traditional LLNA sensitizers and nonsensitizers.

Of the nine substances that produced SI values between 1.8 and 2.5 (**Table C-VII-1)**, four are nonsensitizers (chlorobenzene, hexane, isopropanol, salicylic acid) and five are sensitizers (3-aminophenol, cobalt chloride, 2-mercaptobenzothiazole, methyl methacrylate, and nickel [II] sulfate hexahydrate) based on traditional LLNA results. Among the four traditional LLNA nonsensitizers, six chemical classes are represented; one substance is classified as both a carboxylic acid and phenol (salicylic acid), one substance is both a halogenated and a cyclic hydrocarbon (chlorobenzene), one substance is an acyclic hydrocarbon (hexane), and one substance is an alcohol (isopropanol). Other characteristics of the nonsensitizers (based on traditional LLNA data) include:

- Three substances are liquids (chlorobenzene, hexane, and isopropanol) and one substance is a solid (salicylic acid).
- Molecular weights range from 60 g/mol for isopropanol, 86 g/mol for hexane, 113 g/mol for chlorobenzene, to 138 g/mol for salicylic acid.
- All four substances are soluble in water.
- The peptide reactivity for chlorobenzene, hexane, and isopropanol is minimal; peptide reactivity information for salicylic acid is not available.
- Hexane and salicylic acid are considered irritants based on data in either mice or humans and isopropanol is considered negative based on data in rabbits; irritancy data for chlorobenzene are not available but irritancy potential is assumed as low based on clinical literature (**Table C-VII-1**).
- Among the five traditional LLNA sensitizers, five chemical classes are represented; one substance is a carboxylic acid (methyl methacrylate), two substances are metals (nickel [II] sulfate hexahydrate and cobalt chloride), one substance is both an amine and a phenol (3-aminophenol), and one substance is a heterocyclic compound (2-mercaptobenzothiazole).

Other characteristics of the substances that are classified as sensitizers by the traditional LLNA include:

- Four substances are solids (3-aminophenol, cobalt chloride, 2-mercaptobenzothiazole, and nickel [II] sulfate hexahydrate) and one substance is a liquid (methyl methacrylate).
- Molecular weights range from 100 g/mol for methyl methacrylate, 109 g/mol for 3-aminophenol, 130 g/mol for cobalt chloride, 155 g/mol for nickel (II) sulfate hexahydrate to 167 g/mol for 2-mercaptobenzothiazole.
- 2-Mercaptobenzothiazole is insoluble in water; the other four substances are soluble in water.
- The peptide reactivity for 2-mercaptobenzothiazole is high and that for 3-aminophenol is minimal; peptide reactivity data for the three other substances are not available.
- The EC3 values for the five substances identified as sensitizers by the traditional LLNA are: 0.6% for cobalt chloride, 1.7% for 2-mercaptobenzothiazole, 3.2% for 3-aminophenol, 4.8% for nickel [II] sulfate hexahydrate, and 90% for methyl methacrylate.
- All five substances are considered nonirritants based on GP data (**Table C-VII-1**).

Table C-VII-1 Indeterminate Results for the LLNA: DA When Multiple Stimulation Index Decision Criteria are Used[1]

Substance Name[2]	Vehicle[3]	LLNA: DA[4]	Traditional LLNA[4]	Skin Irritant?
Chlorobenzene	AOO	2.44, 25% (1/1 tests)	- (1.7, 25%)[5]	No data. Low irritancy potential assumed based on clinical literature.
Hexane	AOO	2.31, 100% (1/1 tests)	- (2.2, 100%)	Irritant at 100% (humans)
Isopropanol	AOO	1.97, 50%[5] (1/11 tests)	- (1.7, 50%)[5]	Negative at 100% (rabbits)
Salicylic acid	AOO	2.00, 25% (1/1 tests)	- (2.4, 25%)	Irritant at 20% aq. (mice)
3-Aminophenol (3.2%)	AOO	2.38, 10%[6] (1/3 tests)	+ (5.7, 10%)	Nonirritant at 5% (GP)
Cobalt chloride (0.6%)	DMSO	2.0, 5% (1/8 tests)	+ (7.2, 5%)	Negative at ≤ 0.5% (GP)
2-Mercaptobenzothiazole (1.7%)	DMF	2.01, 50%[5] (1/1 tests)	+ (8.6, 10%)	Nonirritant at 10% (GP)
Methyl methacrylate (90%)	AOO	1.81, 100% (1/1 tests)	+ (3.6, 100%)	Nonirritant at 3 M (GP)
Nickel (II) sulfate hexahydrate (4.8%)	DMSO	2.13, 10% 2.17, 5%[7] (2/8 tests)	+ (3.1, 5%)	Nonirritant at 0.15% (GP); irritant at 10% (humans)

Abbreviations: AOO = acetone: olive oil (4:1); aq. = aqueous; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

"+" = Sensitizer.

"-" = Nonsensitizer.

[1] Data source(s) indicated in Annex III of the BRD.

[2] Numbers in parentheses are EC3 values (concentrations needed to produce a stimulation index [SI] of three) for substances that are sensitizers in the traditional LLNA (see Table C-2 of the BRD).

[3] Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.

[4] Numbers indicated are highest SI and maximum concentration tested; highest SI is at maximum concentration tested, unless otherwise noted.

[5] Highest SI occurred at concentration 10%.

[6] Highest SI occurred at concentration 3%.

[7] Highest SI occurred at concentration 2.5%.

3.0 Test Method Reliability

An assessment of test method reliability (intralaboratory repeatability and intra- and interlaboratory reproducibility) is an essential element of any evaluation of the performance of an alternative test method (ICCVAM 2003). Repeatability refers to the closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under

identical conditions within a given time period (ICCVAM 1997, 2003). Intralaboratory reproducibility refers to the extent to which qualified personnel within the same laboratory can replicate results using a specific test protocol at different times. Interlaboratory reproducibility refers to the extent to which different laboratories can replicate results using the same protocol and test substances, and indicates the extent to which a test method can be transferred successfully among laboratories. With regard to the LLNA: DA test method, there are no known intralaboratory repeatability studies, which was also the situation with the traditional LLNA.

The LLNA: DA data were amenable to both intralaboratory and interlaboratory reproducibility analyses. The evaluation of multiple SI decision criteria in **Section 2.0** of this Annex evaluated SI ≥ 2.5 as the decision criterion for classifying substances as sensitizers when used with a decision criterion of SI ≤ 1.8 to identify nonsensitizers. Thus, this section provides an assessment of reproducibility for the decision criterion of SI ≥ 2.5 to identify sensitizers.

3.1 Intralaboratory Reproducibility

Idehara et al. (2008) evaluated intralaboratory reproducibility of EC3 values for the LLNA: DA using two substances (isoeugenol and eugenol) that were each tested in three different experiments (**Table C-VII-2**). The data indicate coefficients of variation (CVs) of 21% and 11% for isoeugenol and eugenol, respectively. The authors state that for both compounds the EC3 values appeared to be close and that for each test substance the SI values for the same concentration were fairly reproducible (Idehara et al. 2008). The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) also determined the intralaboratory reproducibility of EC2.5 values (estimated concentrations needed to produce an SI of 2.5) for the same set of data. The results for EC2.5 values indicate slightly larger intralaboratory variability compared to EC3 values with CVs of 33% and 13% for isoeugenol and eugenol, respectively.

Table C-VII-2 Intralaboratory Reproducibility of EC3 and EC2.5 Values Using the LLNA: DA[1]

Isoeugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
Vehicle (AOO)	1.00 ± 0.54	1.00 ± 0.54	1.00 ± 0.30
0.5	1.50 ± 0.54	-------	1.22 ± 0.13
1	2.28 ± 0.60	-------	2.77 ± 1.01
2.5	2.78 ± 0.17	3.11 ± 1.15	3.01 ± 0.98
5	3.39 ± 0.69	4.39 ± 1.25	-------
10	5.68 ± 1.19	6.77 ± 0.23	-------
EC3	3.40%	2.35%	2.46%
EC2.5	0.82%	1.37%	0.75%
Mean EC3: 2.74% ± 0.58% and 21% CV *Mean EC2.5: 1.46% ± 0.48% and 33% CV*			

continued

Table C-VII-2 Intralaboratory Reproducibility of EC3 and EC2.5 Values Using the LLNA: DA[1] (continued)

Eugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
Vehicle (AOO)	1.00 ± 0.17	1.00 ± 0.17	1.00 ± 0.09
5	2.92 ± 1.00	2.80 ± 1.08	3.24 ± 0.70
10	7.35 ± 2.62	4.47 ± 0.98	4.79 ± 0.94
25	10.92 ± 3.63	5.62 ± 3.20	7.07 ± 0.44
EC3	5.09%	5.59%	4.50%
EC2.5	4.33%	3.59%	2.87%
Mean EC3: 5.06% ± 0.55% and 11% CV *Mean EC2.5: 4.23% ± 0.57% and 13% CV*			

Abbreviations: AOO = acetone: olive oil (4:1); CV = coefficient of variation; EC2.5 = estimated concentration needed to produce a stimulation index of 2.5; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] Based on results discussed in Idehara et al. 2008; the number per group was not specified.

[2] Mean stimulation index value ± standard deviation.

3.2 Interlaboratory Reproducibility

Furthermore, data were submitted to NICEATM (Annex IV of the BRD) from a two-phased interlaboratory validation study on the LLNA: DA test method (Omori et al. 2008). In the first phase of the interlaboratory validation study, a blinded test of 12 substances was conducted in 10 laboratories. Three substances (2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, and isopropanol) were tested in all 10 laboratories. The remaining nine substances were randomly assigned to subsets of three of the 10 laboratories **(Table C-VII-3)**. In each laboratory, each substance was tested one time at three different concentrations. The dose levels for each substance were predetermined (i.e., the participating laboratories did not determine their own dose levels for testing). Nine substances are sensitizers and three substances are nonsensitizers according to traditional LLNA results. Six substances are reference substances included in LLNA performance standards recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM): cobalt chloride, 2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, isoeugenol, isopropanol, and methyl salicylate.

The second phase of the interlaboratory validation study was designed to evaluate the reliability of the LLNA: DA for testing metallic salts using dimethylsulfoxide (DMSO) as a vehicle since two metals dissolved in DMSO (cobalt chloride and nickel [II] sulfate hexahydrate) from the first phase of the interlaboratory validation study yielded inconsistent results. Five coded substances (two of the five substances were unique to the second phase of the interlaboratory validation study) were tested in seven laboratories **(Table C-VII-4)**. One substance (i.e. hexyl cinnamic aldehyde) was tested in all seven laboratories. The remaining four substances (cobalt chloride, nickel (II) sulfate hexahydrate, lactic acid, and potassium dichromate) were randomly assigned to subsets of four of the seven laboratories. Each laboratory tested the substance one time at three different dose levels. Again, the dose levels for each substance were predetermined. Of the two substances not previously tested in the first phase of the interlaboratory validation study (lactic acid and potassium dichromate), one is a nonsensitizer and the other is a sensitizer according to traditional LLNA results, respectively. In addition, lactic acid is an ICCVAM-recommended LLNA performance standards reference substance.

The LLNA: DA test results from the two-phased interlaboratory validation studies are amenable to interlaboratory reproducibility analyses for three endpoints: sensitizer (positive) or nonsensitizer (negative) classification, and EC2.5 values. Analyses of interlaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer) (**Section 3.2.1**) and a CV analysis for the quantitative results (EC2.5 values) (**Sections 3.2** and **3.3**).

Table C-VII-3 Substances and Allocation for the First Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	Vehicle	Concentration Tested (%)			Laboratory									
					1	2	3	4	5	6	7	8	9	10
2,4-Dinitro-chlorobenzene (+)	AOO	0.03	0.10	0.30	X	X	X	X	X	X	X	X	X	X
Hexyl cinnamic aldehyde (+)	AOO	5	10	25	X	X	X	X	X	X	X	X	X	X
Isopropanol (-)	AOO	10	25	50	X	X	X	X	X	X	X	X	X	X
Abietic acid (+)	AOO	5	10	25		X				X	X			
3-Aminophenol (+)	AOO	1	3	10	X		X					X		
Dimethyl isophthalate (-)	AOO	5	10	25	X		X				X			
Isoeugenol (+)	AOO	1	3	10				X	X				X	
Methyl salicylate (-)	AOO	5	10	25			X				X			X
Formaldehyde (+)	ACE	0.5	1.5	5.0	X	X			X					
Glutaraldehyde (+)	ACE	0.05	0.15	0.50	X	X			X					
Cobalt chloride[2] (+)	DMSO	0.3	1.0	3.0				X		X		X		
Nickel (II) sulfate hexahydrate (+)	DMSO	1	3	10				X		X		X		

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

Table C-VII-4 Substances and Allocation for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	Vehicle	Concentration Tested (%)			Laboratory						
					11	12	13	14	15	16	17
Hexyl cinnamic aldehyde (+)	AOO	5	10	25	X	X	X	X	X	X	X
Cobalt chloride[2] (+)	DMSO	1	3	5	X		X	X			X
Lactic acid (-)	DMSO	5	10	25	X		X		X	X	
Nickel (II) sulfate hexahydrate (+)	DMSO	1	3	10	X	X		X		X	
Potassium dichromate (+)	DMSO	0.1	0.3	1.0	X	X			X		X

Abbreviations: AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

3.2.1 Interlaboratory Reproducibility – Qualitative Results

The qualitative (positive/negative) interlaboratory concordance analysis for the 12 substances that were tested during the first phase of the LLNA: DA interlaboratory validation study is shown in **Table C-VII-5** for SI ≥ 2.5. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), ten substances tested in either three or 10 laboratories had consistent results leading to 100% (3/3 or 10/10) interlaboratory concordance for those substances. There were two discordant substances (3-aminophenol and nickel [II] sulfate hexahydrate) for which interlaboratory concordance was 67% (2/3). One of the three laboratories that tested 3-aminophenol reported SI ≥ 2.5 at the highest dose tested (SI = 2.83 at 10%) and two laboratories did not achieve SI ≥ 2.5 at any dose tested (Annex IV of the BRD). One of the three laboratories that tested nickel (II) sulfate hexahydrate reported a maximum SI = 1.52, while the other two laboratories produced an SI ≥ 2.5 at all three doses tested (Annex IV of the BRD). Notably, when analyzing the dose response curves for the three tests performed for nickel (II) sulfate in the first phase of the two-phased interlaboratory validation study, only one study demonstrated a sufficient dose response (i.e., a parallel increase in SI relative to increase in concentration). Since the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), there were no traditional LLNA concordance data for comparison with the LLNA: DA concordance data from the first phase of the interlaboratory validation study.

Table C-VII-5 Qualitative Results for the First Phase of the Interlaboratory Validation Studies for the LLNA: DA (SI ≥ 2.5)

Substance Name[1]	Qualitative Results (Maximum SI)[2]										Concordance
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	
2,4-Dinitrochlorobenzene (+)	+ (11.97)	+ (9.23)	+ (9.96)	+ (8.53)	+ (7.86)	+ (15.14)	+ (13.18)	+ (12.60)	+ (10.89)	+ (4.71)	10/10
Hexyl cinnamic aldehyde (+)	+ (5.78)	+ (4.82)	+ (4.44)	+ (5.11)	+ (3.97)	+ (5.50)	+ (7.09)	+ (10.22)	+ (3.88)	+ (3.51)	10/10
Isopropanol (-)	- (1.54)	- (0.91)	- (1.01)	- (1.57)	- (0.76)	- (1.97)	- (1.45)	- (1.21)	- (0.70)	- (1.25)	10/10
Abietic acid (+)		+ (4.64)				+ (7.96)	+ (3.98)				3/3
3-Aminophenol (+)	**+ (2.83)**		**- (1.76)**					**- (2.38)**			**2/3**
Dimethyl isophthalate (-)	- (1.34)		- (1.29)				- (1.26)				3/3
Isoeugenol (+)				+ (6.11)	+ (5.54)				+ (7.09)		3/3
Methyl salicylate (-)			- (1.55)				- (1.77)			- (0.83)	3/3
Formaldehyde (+)	+ (4.84)	+ (3.18)			+ (2.69)						3/3
Glutaraldehyde (+)	+ (5.00)	+ (3.39)			+ (2.57)						3/3
Cobalt chloride[3] (+)				+[4] (2.66)		+ (20.55)		+ (8.07)			3/3
Nickel (II) sulfate hexahydrate (+)				**-[5] (1.52)**		**+ (11.78)**		**+[5] (3.49)**			**2/3**

Bolded substances did not achieve 100% interlaboratory concordance.

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] (+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests.

[3] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

[4] Data not reported for the highest dose (3%), only for 0.3% and 1%.

[5] Insufficient dose response.

The qualitative (positive/negative) interlaboratory concordance analysis for the five substances that were tested during the second phase of the LLNA: DA interlaboratory validation study is shown in **Table C-VII-6**. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), four substances (hexyl cinnamic aldehyde, lactic acid, nickel [II] sulfate hexahydrate, and potassium dichromate) tested in either four or seven laboratories had consistent results leading to 100% (4/4 or 7/7) interlaboratory concordance for those substances. There was one discordant substance (cobalt chloride) for which interlaboratory concordance was 75% (3/4). One of the four laboratories that tested cobalt chloride did not report a maximum SI ≥ 2.5 at any dose, while the other three laboratories produced an SI ≥ 2.5 at the highest dose tested. Cobalt chloride was also tested in the first phase of the interlaboratory validation study where interlaboratory concordance was 100% (3/3). Furthermore, as mentioned previously, the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), and therefore there were no traditional LLNA concordance data for comparison with the LLNA: DA concordance data from the second phase of the interlaboratory validation study.

Table C-VII-6 Qualitative Results for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 2.5)

Substance Name[1]	Qualitative Results (Maximum SI)[2]							Concordance
	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17	
Hexyl cinnamic aldehyde (+)	+ (4.47)	+ (5.71)	+ (5.41)	+ (7.60)	+ (3.92)	+ (8.42)	+ (6.45)	7/7
Cobalt chloride[3] (+)	**- (2.01)**		**+ (2.54)**	**+ (4.25)**			**+ (5.06)**	**3/4**
Lactic acid (-)	- (0.93)		- (0.99)		- (0.97)	- (0.91)		4/4
Nickel (II) sulfate hexahydrate (+)	- (0.79)	- (1.24)		- (2.13)		- (1.56)		4/4
Potassium dichromate (+)	+ (4.78)	+ (4.08)			+ (6.01)		+ (6.37)	4/4

Bolded substance did not achieve 100% interlaboratory concordance.

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] (+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests.

[3] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

3.2.2 Interlaboratory Reproducibility – EC2.5 Values

The quantitative (i.e., EC2.5 value) data for interlaboratory reproducibility analysis were obtained from the LLNA: DA results that yielded positive results (i.e., SI ≥ 2.5) during the first and second phase of the LLNA: DA interlaboratory validation study. The equation used for calculating EC2.5 values for the positive results was modified based on the method of linear interpolation reported by Gerberick et al. (2004) for the EC3 value:

$$EC2.5 = c + \left[\frac{(2.5 - d)}{(b - d)}\right] \times (a - c)$$

where the data points lying immediately above and below the SI = 2.5 on the dose response curve have the coordinates of (a, b) and (c, d), respectively (Gerberick et al. 2004). For substances for which the lowest concentration tested resulted in an SI > 2.5, an EC2.5 value was extrapolated according to the equation:

$$EC2.5_{ex} = 2^{\left\{\log_2(c) + \frac{(2.5-d)}{(b-d)} \times \left[\log_2(a) - \log_2(c)\right]\right\}}$$

where the point with the higher SI is denoted with the coordinates of (a, b) and the point with the lower SI is denoted (c, d) (Gerberick et al. 2004).

The EC2.5 values from each laboratory were used to calculate CV values for each substance. The resulting values for the first and second phase of the interlaboratory validation study are shown in **Tables C-VII-7** and **C-VII-8**, respectively. In the first phase of the interlaboratory validation study, CV values ranged from 26% (hexyl cinnamic aldehyde) to 133% (cobalt chloride) and the mean CV was 79% (**Table C-VII-7**). In the second phase of the interlaboratory validation study, CV values ranged from 20% (hexyl cinnamic aldehyde) to 92% (cobalt chloride) and the mean CV was 62% (**Table C-VII-8**).

The ICCVAM-recommended LLNA performance standards indicate that interlaboratory reproducibility should be evaluated with at least two sensitizing chemicals with well-characterized activity in the traditional LLNA. Acceptable reproducibility is attained when each laboratory obtains ECt values (estimated concentrations needed to produce an SI of a specified threshold) within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20% for hexyl cinnamic aldehyde (ICCVAM 2009). In the first phase of the interlaboratory validation study, five laboratories reported EC2.5 values outside the acceptance range indicated for 2,4-dinitrochlorobenzene; two of the five laboratories obtained EC2.5 values that were lower than the specified acceptance range (<0.025%) and three of the five laboratories obtained EC2.5 values that were higher than the specified acceptance range (>0.1%) (**Table C-VII-7**). For hexyl cinnamic aldehyde, all the laboratories obtained an EC2.5 value within the acceptance range (5% to 20%). In the second phase of the interlaboratory validation study, only hexyl cinnamic aldehyde was tested and all seven laboratories obtained EC2.5 values that were within the acceptance range indicated (**Table C-VII-8**).

Table C-VII-7 EC2.5 Values from the First Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name	EC2.5 (%)										Mean EC2.5 (%) ± SD	CV (%)
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10		
2,4-Dinitrochloro-benzene (+)	**0.026 (11.97)**	**0.063 (9.23)**	**0.039 (9.96)**	**0.022 (8.53)**	**0.112 (7.86)**	**0.025 (15.14)**	**0.011 (13.18)**	**0.039 (12.60)**	**0.023 (10.89)**	**0.131 (4.71)**	**0.049 ± 0.041**	**84**
Hexyl cinnamic aldehyde (+)	**8.473 (5.78)**	**9.414 (4.82)**	**11.402 (4.44)**	**7.900 (5.11)**	**14.594 (3.97)**	**10.759 (5.50)**	**6.778 (7.09)**	**7.032 (10.22)**	**12.530 (3.88)**	**9.135 (3.51)**	**9.802 ± 2.510**	**26**
Isopropanol (-)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Abietic acid (+)		6.418				6.469	11.525				8.137 ± 2.934	36
3-Aminophenol (+)	5.471		NA					NA			5.471 ± NA	NA
Dimethyl isophthalate (-)	NA		NA				NA				NA	NA
Isoeugenol (+)				0.657	5.191				0.874		2.240 ± 2.557	114
Methyl salicylate (-)			NA				NA			NA	NA	NA
Formaldehyde (+)	0.393	1.105			4.179						1.892 ± 2.012	106
Glutaraldehyde (+)	0.091	0.351			0.296						0.246 ± 0.137	56
Cobalt chloride[2] (+)				0.822[3]		0.047		0.104			0.325 ± 0.432	133
Nickel (II) sulfate hexahydrate (+)				NA		0.352		IDR			0.352	NA

Boldface indicates substances that are ICCVAM-recommended murine local lymph node assay (LLNA) performance standards reference substances for evaluating interlaboratory reproducibility (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For both 2,4-dinitrochlorobenzene and hexyl cinnamic aldehyde, the highest SI values achieved were from the highest dose tested (0.3% for 2,4-dinitrochlorobenzene and 25% for hexyl cinnamic aldehyde). Shading shows EC2.5 values (estimated concentration needed to produce a stimulation index of 2.5) that are outside of the acceptable range indicated in the ICCVAM-recommended LLNA performance standards: 5-20% for hexyl cinnamic aldehyde and 0.025-0.1% for 2,4-dinitrochlorobenzene.

Abbreviations: CV = coefficient of variation; IDR = insufficient dose response; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; NA = not applicable; SD = standard deviation.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

[3] Data not reported for the highest dose (3%), only for 0.3% and 1%.

Table C-VII-8 EC2.5 Values from the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	EC2.5 (%)							Mean EC2.5 (%) ± SD	CV (%)
	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17		
Hexyl cinnamic aldehyde (+)	**7.737 (4.47)**	**7.374 (5.71)**	**6.772 (5.41)**	**6.361 (7.60)**	**9.902 (3.92)**	**5.366 (8.42)**	**6.783 (6.45)**	**7.185 ±1.417**	**20**
Cobalt chloride[2] (+)	NA		4.111	1.202			0.699	2.004 ±1.842	92
Lactic acid (-)	NA		NA		NA	NA		NA	NA
Nickel (II) sulfate hexahydrate (+)	NA	NA		NA		NA		NA	NA
Potassium dichromate (+)	0.372	0.269			0.087		0.063	0.198 ±0.148	75

Bolded text indicates a substance that is an ICCVAM-recommended murine local lymph node assay (LLNA) performance standards reference substance for evaluating interlaboratory reproducibility (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For hexyl cinnamic aldehyde, the highest SI values achieved were from the highest dose tested (25%). None of the EC2.5 values (estimated concentrations needed to produce a stimulation index of 2.5) are outside of the acceptable range indicated in the ICCVAM-recommended LLNA performance standards (5-20% for hexyl cinnamic aldehyde).

Abbreviations: CV = coefficient of variation; NA = not applicable; SD = standard deviation.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

The interlaboratory CV values for both the first and second phases of the interlaboratory validation study for the LLNA: DA EC2.5 values were higher than that for the traditional LLNA EC3 values. The analysis of interlaboratory variation of EC3 values for the traditional LLNA reported CV values of 6.8% to 83.7% for five substances tested in five laboratories (**Table C-VII-9**; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: DA (hexyl cinnamic aldehyde, 2,4-dinitrochlorobenzene, and isoeugenol). All interlaboratory CV values for the LLNA: DA were greater than that for the traditional LLNA. The CV of 84% for 2,4-dinitrochlorobenzene was greater than the two CV values of 37.4% and 27.2% (which were calculated from five values each), reported by ICCVAM (1999). The CV of 26% and 20% for hexyl cinnamic aldehyde tested in the first and second phase of the LLNA: DA interlaboratory validation study, respectively, were both greater than the 6.8% reported by ICCVAM (1999). The CV of 114% for isoeugenol tested in the LLNA: DA was greater than the 41.2% reported by ICCVAM (1999).

Table C-VII-9 Interlaboratory Reproducibility of the EC3 for Substances Tested in the Traditional LLNA[1]

Substance Name	EC3 (%)					CV (%)
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	
2, 4-Dinitrochlorobenzene	0.3	0.5	0.6	0.9	0.6	37.4
	0.5	0.6	0.4	0.6	0.3	27.2
Hexyl cinnamic aldehyde	7.9	7.6	8.4	7.0	8.1	6.8
Isoeugenol	1.3	3.3	1.8	3.1	1.6	41.2
Eugenol	5.8	14.5	8.9	13.8	6.0	42.5
SLS	13.4	4.4	1.5	17.1	4.0	83.7

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA = murine local lymph node assay; SLS = sodium lauryl sulfate.

[1] From ICCVAM 1999 report.

3.3 Reproducibility for the LLNA: DA Accuracy Analysis Using Multiple Stimulation Index Decision Criteria

Section 2.0 of this annex details the accuracy analysis for the LLNA: DA (using the most prevalent outcome for substances with multiple tests) when using two decision criteria for LLNA: DA results: one criterion to classify substances as sensitizers (SI ≥ 2.5) and one criterion to classify substances as nonsensitizers (SI ≤ 1.8). SI ≥ 2.5 was evaluated for classifying sensitizers because it resulted in no false positives, and SI ≤ 1.8 was evaluated for classifying substances as nonsensitizers because it resulted in no false negatives, with respect to traditional LLNA data. This section evaluates reproducibility of the concordance with the traditional LLNA results by examining the frequency with which SI values in the validation database of 44 substances occurred in one of three SI categories. The three SI categories were:

- SI ≤ 1.8 for classifying nonsensitizers
- 1.8 < SI < 2.5, the range of uncertainty with respect to classification by the traditional LLNA
- SI ≥ 2.5 to classify substances as sensitizers

The validation database for the LLNA: DA consists of 123 tests of 44 substances. The maximum SI achieved by each test and the traditional LLNA outcome (sensitizer vs. nonsensitizer) were used to determine the frequency of the maximum SI. **Table C-VII-10** shows the proportion of sensitizers and nonsensitizers, according to the traditional LLNA for each SI category. Eighty-five percent of the tests (28/33) that yielded SI ≤ 1.8 were for substances that were classified as nonsensitizers by the traditional LLNA; 15% of the tests (5/33) that yielded SI ≤ 1.8 were for substances that were classified as sensitizers by the traditional LLNA. Sixty percent (6/10) of the tests that yielded 1.8 < SI < 2.5 were for substances that were classified as sensitizers by the traditional LLNA. Two tests produced SI values near either end of this range (i.e., near SI = 1.8 or SI = 2.5). The one test for methyl methacrylate produced SI = 1.81 and the one chlorobenzene test produced SI = 2.44. The remainder of the tests in this category, 40% (4/10), were classified as nonsensitizers by the traditional LLNA. One hundred percent (80/80) of the tests that yielded SI ≥ 2.5 were for substances that were classified as sensitizers by the traditional LLNA and 0% (0/80) were classified as nonsensitizers.

Table C-VII-10 Frequency of Maximum SI for LLNA: DA Tests by Category and Traditional LLNA Outcome

Classification Based on Traditional LLNA	Classification Concordance with Traditional LLNA[1]			
	Maximum SI ≤ 1.8	1.8 < Maximum SI < 2.5	Maximum SI ≥ 2.5	Total
Sensitizer	5 (15%)	6 (60%)	80 (100%)	91
Nonsensitizer	28 (85%)	4 (40%)	0 (0%)	32
Total	33	10	80	123

Abbreviations: LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] Numbers shown reflect number of tests. Includes all tests of substances that were tested multiple times. Percentage in parentheses reflects percentage of the total number of tests for each SI category.

The 123 tests evaluated in **Table C-VII-10** include multiple tests for 14 substances. For the 14 substances, three to 18 tests were available. **Table C-VII-11** shows the proportion of the tests for each substance that produced SI values in each category. For the four nonsensitizers with multiple test results, there were 23 tests that produced SI ≤ 1.8 and one test that produced an SI between 1.8 and 2.5. For the 10 sensitizers with multiple test results, however, SI values occurred in all three SI categories. The results for nickel (II) sulfate hexahydrate were particularly variable: 50% (4/8) produced SI ≤ 1.8 (i.e., four tests with SI = 0.79, 1.24, 1.52, and 1.56), 25% (2/8) produced 1.8 < SI < 2.5 (SI = 2.13 and 2.17), and 25% (2/8) produced SI ≥ 2.5 (SI = 3.49 and 11.78). 3-Aminophenol also produced SI values in all three categories: 33% (1/3) of the tests had SI ≤ 1.8 (SI = 1.76), 33% (1/3) of the tests had 1.8 < SI < 2.5 (SI = 2.38), and 33% (1/3) of the tests had SI ≥ 2.5 (SI = 2.83). Cobalt chloride tests produced SI values in two categories: 12.5% (1/8) of the tests had 1.8 < SI < 2.5 (SI = 2.01) and seven of eight tests (87.5%) produced SI ≥ 2.5 (SI = 2.54, 2.66, 3.64, 4.25, 5.06, 8.07, and 20.55). The multiple test results for the remaining seven traditional LLNA sensitizers were 100% concordant (**Table C-VII-11**).

Table C-VII-11 Concordance of LLNA: DA Tests for Substances with Multiple Tests by Maximum SI Category

Substance Name	Maximum SI ≤ 1.8[1]	1.8 < Maximum SI < 2.5[1]	Maximum SI ≥ 2.5[1]	Total
Sensitizers[2]				
Abietic acid	0 (0%)	0 (0%)	4 (100%)	4
3-Aminophenol	1 (33%)	1 (33%)	1 (33%)	3
Cobalt chloride	0 (0%)	1 (12.5%)	7 (87.5%)	8
2,4-Dinitrochlorobenzene	0 (0%)	0 (0%)	11 (100%)	11
Formaldehyde	0 (0%)	0 (0%)	4 (100%)	4
Glutaraldehyde	0 (0%)	0 (0%)	4 (100%)	4
Hexyl cinnamic aldehyde	0 (0%)	0 (0%)	18 (100%)	18
Isoeugenol	0 (0%)	0 (0%)	4 (100%)	4
Nickel (II) sulfate hexahydrate	4 (50%)	2 (25%)	2 (25%)	8
Potassium dichromate	0 (0%)	0 (0%)	5 (100%)	5

continued

Table C-VII-11 Concordance of LLNA: DA Tests for Substances with Multiple Tests by Maximum SI Category (continued)

Substance Name	Maximum SI ≤ 1.8[1]	1.8 < Maximum SI < 2.5[1]	Maximum SI ≥ 2.5[1]	Total
Nonsensitizers[2]				
Dimethyl isophthalate	4 (100%)	0 (0%)	0 (0%)	4
Isopropanol	10 (91%)	1 (9%)	0 (0%)	11
Lactic acid	5 (100%)	0 (0%)	0 (0%)	5
Methyl salicylate	4 (100%)	0 (0%)	0 (0%)	4

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] Numbers shown reflect number of tests. Percentage in parentheses reflects percentage of the total number of tests for each substance.

[2] According to traditional murine local lymph node assay results.

4.0 The Impact of Increasing the LLNA: DA Sample Size on the Substances in the Range of Uncertainty

This section examines the impact of increasing the number of animals used in each LLNA: DA control and treatment group (i.e., sample size) on the size of the range of uncertainty (between 1.8 and 2.5) and on the number of substances in the range of uncertainty.

Since the LLNA: DA accuracy analyses were generally based on tests with four animals per dose group, additional analyses were performed in order to determine if the sample size per dose group contributed to these indeterminate classifications. As detailed below, increasing the sample size for each dose group is unlikely to impact either the number of substances classified as uncertain or the SI values that define the range.

Table C-VII-12 shows the 44 substances evaluated along with their LLNA: DA maximum SI values for each test and corresponding traditional LLNA results. Based on the LLNA: DA maximum SI values, 27 substances had tests with SI ≥ 2.5 (i.e., LLNA: DA sensitizers), eight had tests with SI ≤ 1.8 (i.e., LLNA: DA nonsensitizers), and nine had tests between 1.8 and 2.5 (i.e., LLNA: DA range of uncertainty). Of the nine substances with LLNA: DA tests in the range of uncertainty, five were sensitizers and four were nonsensitizers in the traditional LLNA.

Table C-VII-12 Distribution of LLNA: DA Maximum SI Data for 44 Substances

Substance Name	LLNA: DA Maximum SI Values[2]	Trad. LLNA Result
LLNA: DA Positive; SI ≥ 2.5 (n = 27)		
2,4-Dinitrochlorobenzene	4.71, 7.10, 7.86, 8.53, 9.23, 9.96, 10.89, 11.97. 12.60, 13.18, 15.14	+
5-Chloro-2-methyl-4-isothiazolin-3-one	7.50	+
Abietic acid	3.98, 4.64, 6.26, 7.96,	+
Benzalkonium chloride	6.68	+
Benzoquinone	3.79	+
Butyl glycidyl ether	4.59	+

continued

Table C-VII-12 Distribution of LLNA: DA Maximum SI Data for 44 Substances (continued)

Substance Name	LLNA: DA Maximum SI Values[2]	Trad. LLNA Result
LLNA: DA Positive; SI ≥ 2.5 (n = 27)		
Cinnamic alcohol	5.66	+
Cinnamic aldehyde	4.73	+
Citral	4.40	+
Diethyl maleate	3.78	+
Ethyl acrylate	4.29	+
Ethylene glycol dimethacrylate	4.45	+
Eugenol	7.07	+
Formaldehyde	2.69, 3.18, 4.84, 5.10	+
Glutaraldehyde	2.57, 3.39, 5.00, 6.45	+
Hexyl cinnamic aldehyde	3.51, 3.88, 3.92, 3.97, 4.44, 4.47, 4.82, 5.11, 5.41, 5.50, 5.71, 5.78, 6.45, 6.47, 7.09, 7.60, 8.42, 10.22	+
Hydroxycitronellal	5.69	+
Imidazolidinyl urea	4.67	+
Isoeugenol	5.54, 6.11, 7.09, 12.36	+
Phenyl benzoate	4.24	+
p-Phenylenediamine	5.14	+
Phthalic anhydride	6.85	+
Potassium dichromate	4.08, 4.78, 5.49, 6.01, 6.37	+
Propyl gallate	4.95	+
Resorcinol	4.33	+
Sodium lauryl sulfate	3.39	+
Trimellitic anhydride	4.96	+
LLNA: DA Negative; SI ≤ 1.8 (n = 8)		
1-Bromobutane	1.65	-
Diethylphthalate	1.09	-
Dimethyl isophthalate	0.89, 1.00, 1.26, 1.34	-
Lactic acid	0.91, 0.93, 0.97, 0.99, 1.06,	-
Methyl salicylate	0.83, 1.20, 1.55, 1.77	-
Nickel (II) chloride	1.30	-
Propylparaben	1.28	-
Sulfanilamide	0.86	-

continued

Table C-VII-12 Distribution of LLNA: DA Maximum SI Data for 44 Substances (continued)

Substance Name	LLNA: DA Maximum SI Values[2]	Trad. LLNA Result
LLNA: DA Range of Uncertainty; 1.8 < SI < 2.5 (n = 9)		
2-Mercaptobenzothiazole	**2.00**	+
3-Aminophenol	1.76, **2.38**, 2.83	+
Chlorobenzene	**2.44**	-
Cobalt chloride	**2.01**, 2.54, 2.66, 3.64, 4.25, 5.06, 8.07, 20.55	+
Hexane	**2.31**	-
Isopropanol	0.70, 0.76, 0.91, 1.01, 1.08, 1.21, 1.25, 1.45, 1.54, 1.57, **1.97**	-
Methyl methacrylate	**1.81**	+
Nickel (II) sulfate hexahydrate	0.79, 1.24, 1.52, 1.56, **2.13, 2.17**, 3.49, 11.78	+
Salicylic acid	**2.00**	-

The bold text indicates LLNA: DA tests with maximum SI values between 1.8 and 2.5.

Abbreviations: LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index; Trad. = traditional.

+ = sensitizer; - = nonsensitizer

[1] Multiple values indicate multiple test results.

Increasing the sample size could effectively move any of the borderline substances into (or out of) the range of uncertainty. Also, changing the sample size could widen or narrow the range of uncertainty interval, and thus either increase or decrease the number of substances in the range of uncertainty.

4.1 Impact of Sample Size on the Size of the Range of Uncertainty

When considering the impact of the number of animals on the range of uncertainty interval (and the number of substances within the range), the two substances that determine the range of uncertainty interval are methyl methacrylate and chlorobenzene. The maximum SI for chlorobenzene is 2.44, based on mean SI results from three animals, which are somewhat variable (SI = 1.32, 2.47, or 3.54). Despite this variability, it is unlikely that additional animals could have reduced the mean for the maximum SI sufficiently for chlorobenzene to be correctly classified as a nonsensitizer. Although the individual animal SI data for methyl methacrylate are unpublished (Idehara unpublished), similarly it would be unlikely that additional animals would have increased the maximum SI sufficiently for it to be correctly classified as a sensitizer.

4.2 Impact of Sample Size on the Number of Substances in the Range of Uncertainty

Within the range of uncertainty two additional substances are problematic in the LLNA: DA (hexane and nickel [II] sulfate hexahydrate). For hexane (traditional LLNA nonsensitizer, despite a maximum SI = 2.31 [average of SI = 2.89, 2.17, 1.87 for the three animals] based on three animals per dose group), it is unlikely that additional animals would have resulted in a mean maximum SI below the lower uncertainty limit of 1.80. In addition, nickel (II) sulfate hexahydrate in many ways is the most problematic substance since it had a wide range of maximum SI values (**Table C-VII-12**). Certainly, additional animals would not help for this substance. Generally, the LLNA: DA does not seem to perform well for these four substances, although the results for three of the substances are based on a single test.

The SI values determined for these 44 substances were generally based on four animals per dose group. The discussion described above indicates that additional animals would likely not have had an appreciable impact on either the number of substances in the range of uncertainty or on the range of uncertainty interval.

5.0 Analysis of Physicochemical Characteristics for Substances in the Range of Uncertainty

5.1 Introduction

The following information is presented to evaluate the use of physicochemical characteristics and other parameters to help identify the sensitization category for substances that are not clearly identified as sensitizers or nonsensitizers in the LLNA: DA when using multiple SI decision criterion to identify sensitizers and nonsensitizers (SI ≥ 2.5 and SI ≤1.8 for sensitizers and nonsensitizers, respectively). Characteristics that distinguish between sensitizers and nonsensitizers may aid in the interpretation of LLNA: DA SI values that fall within the range of uncertainty between 1.8 and 2.5.

The physicochemical information evaluated included peptide reactivity, molecular weight, octanol/water partition coefficient, physical form, and chemical class. The other parameters evaluated were vehicle control substance and potential local skin irritation (i.e., whether the substance was tested at a potentially irritating concentration). The "potentially irritating" concentration is based on either (1) skin irritation with the concentration tested based on hazard identification (e.g., European Centre for Ecotoxicology and Toxicology of Chemicals skin irritation database; published traditional LLNA studies that provided skin irritation data), or (2) the concentration tested in the LLNA exceeded the challenge concentration used in the guinea pig maximization test (GPMT) (i.e., the maximum nonirritating concentration is used for challenge in the GPMT). The information used for this analysis is provided in **Annex VIIa** unless otherwise noted.

5.2 LLNA: DA Data

The substances evaluated were the 44 substances used in the LLNA: DA accuracy analysis (32 sensitizers and 12 nonsensitizers, according to traditional LLNA results) (see Section 6.0 of the BRD). **Table C-VII-13** lists the nine substances in the LLNA: DA range of uncertainty, between 1.8 and 2.5, along with the LLNA: DA maximum SI values and the traditional LLNA results. Based on the traditional LLNA, five substances were sensitizers and four substances were nonsensitizers.

Table C-VII-13 Substances with Tests in the Range of Uncertainty: 1.8 < SI < 2.5

Substance Name	Maximum SI LLNA: DA[1]	Maximum SI Traditional LLNA	Traditional LLNA Result
Isopropanol	0.70, 0.76, 0.91, 1.01, 1.08, 1.21, 1.25, 1.45, 1.54, 1.57, **1.97**	1.7	-
Chlorobenzene	**2.44**	1.7	-
Hexane	**2.31**	2.2	-
Salicylic acid	**2.00**	2.5	-
Nickel (II) sulfate hexahydrate	0.79, 1.24, 1.52, 1.56, **2.13, 2.17**, 3.49, 11.78	3.1	+
Methyl methacrylate	**1.81**	3.6	+

continued

Table C-VII-13 Substances with Tests in the Range of Uncertainty: 1.8 < SI < 2.5 (continued)

Substance Name	Maximum SI LLNA: DA[1]	Maximum SI Traditional LLNA	Traditional LLNA Result
3-Aminophenol	1.76, **2.38**, 2.83	5.7	+
Cobalt chloride	**2.01**, 2.54, 2.66, 3.64, 4.25, 5.06, 8.07, 20.55	7.2	+
2-Mercaptobenzothiazole	**2.00**	8.6	+

The bold text indicates LLNA: DA tests with maximum SI values between 1.8 and 2.5.

Abbreviations: LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

+ = Sensitizer; - = Nonsensitizer

[1] Multiple values indicate multiple test results.

5.3 Peptide Reactivity

Because the ability to form stable conjugates with protein is a key requirement for a substance to produce skin sensitization, peptide reactivity information may assist in determining sensitization potential (Jowsey et al. 2006).

5.3.1 Categorical Analysis

Gerberick et al. (2007) classified peptide reactivity as high, moderate, low, or minimal based on a classification tree model used to relate the depletion of cysteine- and lysine-containing peptides to relative skin sensitization potency categories from Kimber et al. (2003) that were based on LLNA EC3 values. The preferred model, which was based on the average of two peptide depletion measurements (one using a cysteine-containing peptide at a 1:10 molar ratio with the test substance and one using a lysine-containing peptide at a 1:50 molar ratio with the test substance), accurately predicted the sensitizer or nonsensitizer outcomes of 89% (72/81) of the substances evaluated. Peptide reactivity information for 26/44 substances evaluated for the LLNA: DA was obtained from Gerberick et al. (2007). Using the peptide reactivity information from Gerberick et al. (2007) provided peptide reactivity classification for 17 sensitizers and nine nonsensitizers. The peptide reactivity categories evaluated for the LLNA: DA were used to analyze the association of the high, moderate, low, or minimal peptide reactivity categories with the traditional LLNA sensitizer and nonsensitizer status of the 26 test substances.

Table C-VII-14 lists the nine substances in the range of uncertainty and the peptide reactivity categories for those that could be obtained from Gerberick et al. (2007)(4/9 substances), and provides the percentage of cysteine depletion (values from Natsch et al. [2009]). **Annex VIIa** shows the peptide reactivity information for all 26 substances for which it was available from Gerberick et al. (2007).

Table C-VII-14 Peptide Reactivity Data for Substances in the Range of Uncertainty

Substance Name	Traditional LLNA Result	Peptide Reactivity Category[1]	% Cysteine Depletion[2]
Isopropanol	-	Minimal	0.3
Chlorobenzene	-	Minimal	0.4
Hexane	-	Minimal	-0.4
Salicylic acid	-	NA	3.3
Nickel (II) sulfate hexahydrate	+	NA	35.5
Methyl methacrylate	+	NA	NA
3-Aminophenol	+	NA	7.0
Cobalt chloride	+	NA	NA
2-Mercaptobenzothiazole	+	High	100

Abbreviations: LLNA = murine local lymph node assay.

+ = Sensitizer; - = Nonsensitizer

[1] Categories from Gerberick et al. (2007).

[2] Values from Natsch et al. (2009).

Table C-VII-15 shows the proportions of the 17 sensitizers and nine nonsensitizers in each category of peptide reactivity. Traditional LLNA nonsensitizers, across all relevant LLNA: DA SI categories (i.e., whether SI ≤ 1.8, or 1.8 < SI < 2.5) were associated with minimal to low peptide reactivity; 100% (9/9) nonsensitizers with peptide reactivity data were in the minimal and low categories. The 17 sensitizers with peptide reactivity data across all relevant LLNA: DA SI categories (i.e., whether 1.8 < SI < 2.5 or SI ≥ 2.5) were generally associated with moderate to high peptide reactivity (76% [13/17]), however, 12% (2/17) of the sensitizers were associated with low peptide reactivity and 12% (2/17) of the sensitizers were associated with minimal peptide reactivity.

Table C-VII-15 Peptide Reactivity for Sensitizers vs. Nonsensitizers[1]

Peptide Reactivity Category[2]	Sensitizer[3]/ LLNA: DA SI ≥ 2.5	Nonsensitizer[3]/ LLNA: DA SI ≤ 1.8	Sensitizer[3]/ 1.8 < LLNA: DA SI < 2.5	Nonsensitizer[3]/ 1.8 < LLNA: DA SI < 2.5
High	56% (9/16)	0% (0/6)	100% (1/1)	0% (0/3)
Moderate	19% (3/16)	0% (0/6)	0% (0/1)	0% (0/3)
Low	13% (2/16)	17% (1/6)	0% (0/1)	0% (0/3)
Minimal	13% (2/16)	83% (5/6)	0% (0/1)	100% (3/3)
NA	11	2	4	1
Total	27	8	5	4

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; NA = peptide reactivity information was not available; SI = stimulation index.

[1] Number of substances shown. Proportion in parentheses based on the total number of substances with peptide reactivity data.

[2] Determined using data in Gerberick et al. (2007).

[3] Based on traditional murine local lymph node assay results.

There are insufficient data to definitively choose a single "breakpoint" for using peptide reactivity to predict sensitizers. However, ranges of reactivity (i.e., low to high vs. minimal; moderate to high vs. minimal to low) appear useful since Fisher's exact test shows that peptide reactivity was highly associated ($p < 0.001$) with the traditional LLNA results using either of these breakpoints (**Table C-VII-16**).

Table C-VII-16 Fisher's Exact Test for Association of Peptide Reactivity with Sensitizers and Nonsensitizers[1]

Peptide Reactivity Category	Sensitizer	Nonsensitizer	Peptide Reactivity Category	Sensitizer	Nonsensitizer
Low to High	15	1	Moderate to High	13	0
Minimal	2	8	Minimal to Low	4	9
$p = 0.0002$ (Fisher's Exact Test)			$p = 0.0004$ (Fisher's Exact Test)		

[1]Number of substances with peptide reactivity information in each category shown.

Either breakpoint shown in **Table C-VII-16** would correctly classify 4/4 substances in the range of uncertainty that have peptide reactivity data. **Table C-VII-15** shows the prediction of sensitizer/nonsensitizer outcomes for 4/4 substances in the range of uncertainty for which peptide reactivity data are available. If moderate to high peptide reactivity were selected to predict sensitizers and minimal to low peptide reactivity was selected to predict nonsensitizers, 100% (1/1) sensitizers with peptide reactivity data would be predicted correctly and 100% (3/3) nonsensitizers would be predicted correctly. If low to high peptide reactivity were selected to predict sensitizers and minimal peptide reactivity was selected to predict nonsensitizers, 100% (1/1) sensitizers with peptide reactivity data would be predicted correctly and 100% (3/3) nonsensitizers would be predicted correctly. Regardless of the breakpoint, the association is highly significant and use of peptide reactivity as a "tie-breaker" variable for those substances for which the LLNA: DA assay produces SI values in the range of uncertainty should be considered.

5.3.2 Numerical Analysis

Peptide reactivity data as percent cysteine depletion were available for 38/44 substances tested in the LLNA: DA. Most of the cysteine depletion data were obtained from Natsch et al. (2009). Natsch et al. (2009) measured peptide depletion with methods similar to Gerberick et al. (2007) using a cysteine-containing peptide at a 1:10 molar ratio with the test substance. Thus, cysteine depletion data was obtained from Gerberick et al. (2007) for substances that were not included in Natsch et al. (2009). Natsch et al. (2009) demonstrated that using >15% cysteine-containing peptide depletion to classify sensitizers yielded an overall accuracy of 80% (93/116). The cysteine depletion data were used to analyze sensitizer/nonsensitizer classification using various peptide depletion cutoff values. Cysteine depletion data were available for 7/9 substances in the range of uncertainty (see **Table C-VII-14**).

The analysis evaluated the performance of several different percent cysteine depletion values by determining the accuracy, false negative rate, and false positive rate for classifying substances as sensitizers or nonsensitizers. The results indicated that highest accuracy (82% [31/38]) occurred when cysteine depletion >5.15% was used to classify substances as sensitizers. At >5.15% cysteine

depletion, the false positive rate was 20% (2/10) and the false negative rate was 18% (5/28). See **Annex VIIb** for the performance of other cysteine depletion cutoffs. **Table C-VII-17** shows that 80% (20/25) of the sensitizers with SI ≥ 2.5, had cysteine depletion values >5.15% and 67% (4/6) of the nonsensitizers with SI ≤ 1.8 had cysteine depletion values <5.15% values. For the substances with 1.8 < SI < 2.5, 100% (3/3) of the sensitizers had cysteine depletion >5.15% and 100% (4/4) of the nonsensitizers had cysteine depletion ≤5.15%. Thus, using a cysteine depletion cutoff of >5.15% to classify sensitizers would have correctly classified 100% of the seven substances, with cysteine depletion data, in the range of uncertainty. This was the same result as using the low to high peptide reactivity categories of Gerberick et al. (2007) to predict sensitizers and the minimal peptide reactivity category to predict nonsensitizers in the range of uncertainty.

Natsch et al. (2009) indicated that at least 15% peptide depletion is needed for significant results in most cases; therefore, the percentage of sensitizers and nonsensitizers associated with peptide depletion ≤15% and >15% were also evaluated (**Table C-VII-17**). The results were similar to the NICEATM-determined cutoff value of 5.15% cysteine depletion; only one substance was classified differently. Using the cysteine depletion cutoff of >15% to classify sensitizers would have correctly classified 100% (4/4) of the sensitizers and 67% (2/3) of the nonsensitizers in the range of uncertainty.

Table C-VII-17 Correct Classification Rate of Sensitizers vs. Nonsensitizers by Cysteine Depletion[1]

Cysteine Depletion Cutoff	Sensitizer[2]/ LLNA: DA SI ≥ 2.5	Nonsensitizer[2]/ LLNA: DA SI ≤ 1.8	Sensitizer[2]/ 1.8 < LLNA: DA SI < 2.5	Nonsensitizer[2]/ 1.8 < LLNA: DA SI < 2.5
≤5.15%	20% (5/25)	67% (4/6)	0% (0/3)	100% (4/4)
>5.15%	80% (20/25)	33% (2/6)	100% (3/3)	0% (0/4)
≤15%	28% (7/25)	100% (6/6)	33% (1/3)	100% (4/4)
>15%	72% (18/25)	0% (0/6)	67% (2/3)	0% (0/4)
NA	2	2	2	0
Total	27	8	5	4

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; NA = peptide reactivity information was not available; SI = stimulation index.

[1] Number of substances shown. Proportion in parentheses based on the total number of substances in the SI category with peptide reactivity data.

[2] Based on traditional murine local lymph node assay results.

5.4 Molecular Weight

The molecular weights of the 32 sensitizers and 12 nonsensitizers (based on traditional LLNA results) were not different, as shown by the means and standard deviations in **Table C-VII-18**. The standard deviations for sensitizers and nonsensitizers have a large range of overlap.

Table C-VII-18 Molecular Weight (g/mol) for Sensitizers vs. Nonsensitizers

	Sensitizer[1]/ LLNA: DA SI ≥ 2.5	**Nonsensitizer[1]/ LLNA: DA SI ≤ 1.8**	**Sensitizer[1]/ 1.8 < LLNA: DA SI < 2.5**	**Nonsensitizer[1] 1.8 < LLNA: DA SI < 2.5**
Mean	174	159.7	132.2	99.3
Standard deviation	75.5	41.4	28.7	33.6
Total	27	8	5	4

Abbreviation: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] Based on traditional murine local lymph node assay results.

5.5 Octanol-Water Partition Coefficient (log K_{ow})

The octanol-water partition coefficients (log K_{ow}) of the 32 sensitizers and 12 sensitizers (based on traditional LLNA results) were not different, as shown by the means and overlapping standard deviations in **Table C-VII-19**. Log K_{ow} values for six substances were unavailable.

Table C-VII-19 Log K_{ow} for Sensitizers vs. Nonsensitizers

	Sensitizer[1]/ LLNA: DA SI ≥ 2.5	**Nonsensitizer[1]/ LLNA: DA SI ≤ 1.8**	**Sensitizer[1]/ 1.8 < LLNA: DA SI < 2.5**	**Nonsensitizer[1]/ 1.8 < LLNA: DA SI < 2.5**
Mean	1.33	1.76	1.32	1.81
Standard deviation	2.77	1.38	1.37	1.39
Total	27[2]	8[3]	5[4]	4

Abbreviation: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] Based on traditional murine local lymph node assay results.

[2] Log K_{ow} was not available for three substances.

[3] Log K_{ow} was not available for one substance.

[4] Log K_{ow} was not available for two substances.

5.6 Physical Form

Table C-VII-20 shows that physical form was not associated with traditional LLNA sensitizer/nonsensitizer outcomes. Solid form seemed to be associated with sensitizer outcomes (80% [4/5]) and liquid form (75% [3/4]) seemed to be associated with nonsensitizer outcomes for the substances in the range of uncertainty, 1.8 < SI < 2.5. This relationship, however, was not consistent for the remaining sensitizers (with SI ≥ 2.5) and nonsensitizers (with SI ≤ 1.8). Thus, physical form cannot be used to accurately classify these substances.

Table C-VII-20 Physical Form for Sensitizers vs. Nonsensitizers[1]

Physical Form	Sensitizer[2]/ LLNA: DA SI ≥ 2.5	Nonsensitizer[2]/ LLNA: DA SI ≤ 1.8	Sensitizer[2]/ 1.8 < LLNA: DA SI < 2.5	Nonsensitizer[2]/ 1.8 < LLNA: DA SI < 2.5
Solids	13 (48%)	5 (63%)	4 (80%)	1 (25%)
Liquids	13 (48%)	3 (37%)	1 (20%)	3 (75%)
Either	1[3] (4%)	0 (0%)	0 (0%)	0 (0%)
Total	27	8	5	4

Abbreviation: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] Number of substances shown. Proportion of total sensitizers or nonsensitizers in the SI category shown in parentheses.

[2] Based on traditional murine local lymph node assay results.

[3] Benzalkonium chloride.

5.7 Vehicle Control Substances

Table C-VII-21 shows the proportions of sensitizers and nonsensitizers (based on traditional LLNA results) for each vehicle control substance used for traditional LLNA and LLNA: DA testing. Because there were too many vehicles with few substances to make an adequate comparison, the substances tested in acetone: olive oil (AOO) were compared with all other vehicles combined. The proportions of sensitizers and nonsensitizers tested in AOO vs. all other vehicles were similar.

Table C-VII-21 Vehicle Control for Sensitizers vs. Nonsensitizers[1]

Vehicle	Sensitizer[2]/ LLNA: DA SI ≥ 2.5	Nonsensitizer[2]/ LLNA: DA SI ≤ 1.8	Sensitizer[2]/ 1.8 < LLNA: DA SI < 2.5	Nonsensitizer[2]/ 1.8 < LLNA: DA SI < 2.5
Acetone: olive oil (4:1)	19 (70%)	5 (63%)	2 (40%)	4 (100%)
Dimethyl formamide	3 (11%)	1 (13%)	1 (20%)	0 (0%)
Acetone	3 (11%)	0 (0%)	0 (0%)	0 (0%)
Dimethyl sulfoxide	1 (4%)	2 (25%)	2 (40%)	0 (0%)
Methyl ethyl ketone	1 (4%)	0 (0%)	0 (0%)	0 (0%)
Total	27	8	5	4
Acetone: Olive Oil vs. Other Vehicles				
Acetone: olive oil (4:1)	19 (70%)	5 (63%)	2 (40%)	4 (100%)
All others	8 (30%)	3 (37%)	3 (60%)	0 (0%)
Total	27	8	5	4

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] Numbers of substances shown. Proportion of total in the SI category shown in parentheses.

[2] Based on traditional murine local lymph node assay results.

5.8 Skin Irritation

The maximum concentrations tested in the traditional LLNA were compared with concentrations known to produce skin irritation to determine whether there was a relationship between skin irritation and sensitizer or nonsensitizer results in the traditional LLNA. For the sensitizers, 81% (26/32) were tested at potentially irritating concentrations, while 75% (9/12) of the nonsensitizers were tested at potentially irritating concentrations. For the entire group of substances tested, 80% (35/44) were tested at irritating concentrations.

5.9 Conclusion

Based on the available data, peptide reactivity is the only promising characteristic for a positive association with LLNA sensitizer/nonsensitizer results that could be used to assist in classifying substances that produce LLNA: DA SI values in the range of uncertainty. While there are insufficient data to definitively choose a single "breakpoint" for using peptide reactivity to predict sensitizers, ranges of peptide reactivity were highly associated ($p < 0.001$) with the traditional LLNA results using either of these breakpoints when compared to LLNA: DA results within the range of uncertainty. By comparison, peptide reactivity was highly associated ($p < 0.001$) with the traditional LLNA results using the "Low to High vs. Minimal" breakpoints. Thus, peptide reactivity could be used as a "tie breaker" for those substances for which the LLNA: DA produces SI values in the range of uncertainty. The numerical analysis using different cysteine depletion cutoffs also supports the conclusion that peptide reactivity is associated with sensitization outcomes.

Annex VIIa Data for Substances Tested Using the LLNA: DA Method

Substance Name	Vehicle	Trad. LLNA SI	LLNA: DA SI and Highest Conc. Tested (%)[1]	MW (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Cys-Depletion (%)[3]	Physical Form	Chemical Class[4]	Skin Irritant[5]	Highest Conc. Tested[6] (%)	Max. Non-Irritating Conc. (%) (unless noted)[7]
2,4-Dinitrochloro-benzene	AOO	43.9	7.10, 11.97, 9.23, 9.96, 8.53, 7.86, 15.14, 13.18, 12.60, 10.89, 4.71, 7.10; (1, 0.3, 0.3, 0.3, 0.3, 0.3, 0.3, 0.3, 0.3, 0.3, 0.3, 1)	202.6	2.27	High	100	Solid	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated; Nitro Compounds	YES	0.25	0.1
5-Chloro-2-methyl-4-isothiazolin-3-one	DMF	27.7	7.50; (0.1)	132.3	0.92	High	94.8	Liquid	Sulfur Compounds, Heterocyclic Compounds	YES	0.1	0.1
Abietic acid	AOO	5.2	6.26, 4.64, 7.96, 3.98; (25)	302.5	6.46	NA	NA	Solid	Hydrocarbons, Cyclic; Polycyclic Compounds	YES	25	2.5
Benzalkonium chloride	ACE	11.1	6.68; (2.5)	170.7	NA	NA	-6.8	Solid/ Liq	Amines; Onium Compounds	YES	2	Irritant at 2% (mice)
Benzoquinone	AOO	52.3	3.79; (0.1)	108.1	1.17	High	91.6	Solid	Quinones	YES	2.5	2.5
Butyl glycidyl ether	AOO	5.6	4.59; (50)	130.2	1.42	NA	84.8	Liquid	Ethers	YES	50	0.1
Cinnamic alcohol	AOO	5.7	5.66; (90)	134.2	2.29	NA	11	Solid	Alcohols	YES	25	1
Cinnamic aldehyde	AOO	18.4	4.73; (15)	132.2	1.82	High	90.5	Liquid	Aldehydes	YES	25	0.75
Citral	AOO	20.5	4.40; (25)	152.2	3.45	NA	34.7	Liquid	Hydrocarbons, Other	YES	20	0.5
Diethyl maleate	AOO	22.6	3.78; (10)	172.2	0.89	High	100	Liquid	Carboxylic Acids	YES	100	100
Ethyl acrylate	AOO	3.98	4.29; (50)	100.1	NA	High	96.4[8]	Liquid	Carboxylic Acids	YES	50	0.3 M
Ethylene glycol dimethacrylate	MEK	7	4.45; (50)	198.2	1.38	High	87.3[8]	Liquid	Carboxylic Acids	YES	50	1

Substance Name	Vehicle	Trad. LLNA SI	LLNA: DA SI and Highest Conc. Tested (%)[1]	MW (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Cys-Depletion (%)[3]	Physical Form	Chemical Class[4]	Skin Irritant[5]	Highest Conc. Tested[6] (%)	Max. Non-Irritating Conc. (%) (unless noted)[7]
Eugenol	AOO	17	7.07; (25)	164.2	2.73	NA	54	Liquid	Carboxylic Acids	YES	50	25
Formaldehyde	ACE	4	5.10, 4.84, 3.18, 2.69; (2.5, 5, 5, 5)	30.0	0.35	Moderate	56.5	Liquid	Aldehydes	NO	1.85	2
Glutaraldehyde	ACE	18	6.45, 5.00, 3.39, 2.57; (0.25, 0.50, 0.50, 0.50)	100.1	-0.18	High	30	Liquid	Aldehydes	NA	2.5	NA
Hexyl cinnamic aldehyde	AOO	20	6.47, 5.78, 4.82, 4.44, 5.11, 3.97, 5.50, 7.09, 10.22, 3.88, 3.51, 4.47, 5.71, 5.41, 7.60, 3.92, 8.42, 6.45; (25)	216.3	4.82	Minimal	-0.3	Liquid	Aldehydes	NO	50	Mild irritant at 100% (rabbits)
Hydroxycitronellal	AOO	8.5	5.69; (50)	172.3	2.11	Low	46.7	Liquid	Hydrocarbons, Other	YES	100	50
Imidazolidinyl urea	DMF	5.5	4.67; (50)	388.3	-8.28	Moderate	46.1	Solid	Urea	NO	50	75
Isoeugenol	AOO	31	12.36, 6.11, 5.54, 7.09; (50, 10, 10, 10)	164.2	2.65	NA	98.4	Liquid	Carboxylic Acids	YES	5	5
Phenyl benzoate	AOO	11.1	4.24; (10)	198.2	2.89	NA	38.5	Solid	Carboxylic Acids	NA	25	NA
p-Phenylenediamine	AOO	26.4	5.14; (1)	108.1	-0.39	NA	95.2	Solid	Amines	YES	1	0.5
Phthalic anhydride	AOO	+NA	5.49; (1)	148.1	2.07	Moderate	-1.9	Solid	Anhydrides; Carboxylic Acids	NA	NA	10
Potassium dichromate	DMSO	33.6	4.78, 4.08, 6.01, 6.37, 5.49; (1)	294.2	-3.59	NA	NA	Solid	Inorganic Chemical, Chromium Compounds; Inorganic Chemical, Potassium Compounds	YES	0.5	0.15

Substance Name	Vehicle	Trad. LLNA SI	LLNA: DA SI and Highest Conc. Tested (%)[1]	MW (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Cys-Depletion (%)[3]	Physical Form	Chemical Class[4]	Skin Irritant[5]	Highest Conc. Tested[6] (%)	Max. Non-Irritating Conc. (%) (unless noted)[7]
Propyl gallate	AOO	33.6	4.95; (2.5)	212.2	NA	High	62.2	Solid	Carboxylic Acids	YES	25	5
Resorcinol	AOO	10.4	4.33; (25)	110.1	1.03	Minimal	1.6	Solid	Phenols	YES	50	Irritant at 15% (humans)
Sodium lauryl sulfate	DMF	8.9	3.39; (10)	288.4	1.69	NA	7.2	Solid	Alcohols; Sulfur Compounds; Lipids	YES	20	Irritant at 20% (rabbits)
Trimellitic anhydride	AOO	4.6	4.96; (0.50)	192.1	1.95	Low	-1.1	Solid	Anhydride; Carboxylic Acids	YES	25	10
2-Mercaptobenzo-thiazole	DMF	8.6	2.00; (50)	167.3	2.86	High	100	Solid	Heterocyclic Compounds	YES	10	10
3-Aminophenol	AOO	5.7	2.83, 1.76, 2.38; (10)	109.1	0.24	NA	7	Solid	Amines; Phenols	YES	10	5
Chlorobenzene	AOO	1.7	2.44; (25)	112.6	2.64	Minimal	0.4[8]	Liquid	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated	NA	25	NA
Cobalt chloride	DMSO	7.2	3.64, 2.66, 20.55, 8.07, 2.01, 2.54, 4.25, 5.06; (5, 1, 3, 3, 5, 5, 5, 5)	129.8	0.85	NA	NA	Solid	Inorganic Chemical, Elements; Inorganic Chemical, Metals	YES	5	0.5
Hexane	AOO	2.2	2.31; (100)	86.2	3.29	Minimal	-0.4[8]	Liquid	Hydrocarbons, Acyclic	YES	100	Irritant at 100% (humans)
Isopropanol	AOO	1.7	1.08, 1.54, 0.91, 1.01, 1.57, 0.76, 1.97, 1.45, 1.21, 0.70, 1.25; (50)	60.1	0.28	Minimal	0.3	Liquid	Alcohols	NO	50	Irritant at 100% (rabbits)
Methyl methacrylate	AOO	3.6	1.81; (100)	100.1	NA	NA	NA	Liquid	Carboxylic Acids	YES	100	3 M

Substance Name	Vehicle	Trad. LLNA SI	LLNA: DA SI and Highest Conc. Tested (%)[1]	MW (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Cys-Depletion (%)[3]	Physical Form	Chemical Class[4]	Skin Irritant[5]	Highest Conc. Tested[6] (%)	Max. Non-Irritating Conc. (%) (unless noted)[7]
Nickel (II) sulfate hexahydrate	DMSO	3.1	2.17, 1.52, 11.78, 3.49, 0.79, 1.24, 2.13, 1.56; (5, 10, 10, 10, 10, 10, 10, 10)	154.8	NA	NA	35.5	Solid	Inorganic Chemical, Elements;	YES	5	0.15
Salicylic acid	AOO	2.5	2.00; (25)	138.1	1.03	NA	3.3	Solid	Phenols; Carboxylic Acids	YES	25	Irritant at 20% (mice)
1-Bromobutane	AOO	1.2	1.65; (25)	137.0	2.65	Low	13.8[8]	Liquid	Hydrocarbons, Halogenated	NA	25	NA
Diethyl phthalate	AOO	1.5	1.09; (100)	222.2	2.65	Minimal	0.8	Liquid	Carboxylic Acids	YES	100	Irritant at 100% (rabbits)
Dimethyl isophthalate	AOO	1	0.89, 1.34, 1.00, 1.26; (25)	194.2	1.66	NA	NA	Solid	Carboxylic Acids	YES	25	10
Lactic acid	DMSO	2.2	1.06, 0.93, 0.99, 0.97, 0.91; (50, 25, 25, 25, 25)	90.1	-0.65	Minimal	2.5	Solid	Carboxylic Acids	YES	25	Slightly irritating at 10% (rabbits)
Methyl salicylate	AOO	2.9	1.20, 1.55, 1.77, 0.83; (25)	152.2	2.60	Minimal	0.3	Liquid	Carboxylic Acids; Phenols	YES	20	Irritant at 10% (mice)
Nickel (II) chloride	DMSO	2.4	1.30; (10)	129.6	NA	NA	NA	Solid	Inorganic Chemical, Elements;	YES	5	0.15
Propylparaben	AOO	1.4	1.28; (25)	180.2	2.98	Minimal	8	Solid	Carboxylic Acids; Phenols	YES	25	10
Sulfanilamide	DMF	1	0.86; (25)	172.2	0.40	Minimal	-1.3[8]	Solid	Hydrocarbons, Cyclic; Sulfur Compounds	YES	50	Irritant at 25% (humans)

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); Conc.= concentration; Cys = cysteine-containing peptide; DMF = *N,N*-dimethyl formamide; DMSO = dimethyl sulfoxide; GP = guinea pig; K_{ow} = octanol/water partition coefficient; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; Max. = maximum; MEK = methyl ethyl ketone; MW = molecular weight; NA = not available; SI = stimulation index; Trad. = traditional.

Shaded cells represent substances for which LLNA: DA test(s) resulted in the highest SI value falling between 1.8 and 2.5.

[1] Highest SI value from LLNA: DA test(s); respective highest concentration tested for each SI value in parentheses.

[2] K_{ow} represents the estimated octanol-water partition coefficient (expressed on log scale) calculated by the Syracuse Research Corporation from the website: http://www.srcinc.com/what-we-do/databaseforms.aspx?id=385.

[3] Peptide reactivity based on cysteine and lysine depletion as reported in Gerberick et al. 2007. Cysteine depletion values are primarily from Natsch et al. (2009) unless otherwise noted.

[4] Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, as developed by the National Library of Medicine: http://www.nlm.nih.gov/mesh/meshhome.html.

[5] Highest concentration tested compared to the maximum non-irritating concentration.

[6] Highest concentration tested in the traditional LLNA.

[7] Guinea pig data unless noted.

[8] Data from Gerberick et al. (2007).

Annex VIIb Performance of Cysteine Depletion Cutoffs for the Prediction of 28 Traditional LLNA Sensitizers and 10 Traditional LLNA Nonsensitizers Tested in the LLNA: DA

Cys Depletion (%)	Accuracy (%)	Sensitivity (%)	Specificity (%)	False Positive (%)	False Negative (%)	Number of Substances Çorrect +	Number of Substances False +	Number of Substances False -	Number of Substances Çorrect -
> -4.350	71	96	0	100	4	27	10	1	0
> -1.600	68	93	0	100	7	26	10	2	0
> -1.200	71	93	10	90	7	26	9	2	1
> -0.7500	68	89	10	90	11	25	9	3	1
> -0.3500	71	89	20	80	11	25	8	3	2
> 0.0	68	86	20	80	14	24	8	4	2
> 0.3500	74	86	40	60	14	24	6	4	4
> 0.6000	76	86	50	50	14	24	5	4	5
> 1.200	79	86	60	40	14	24	4	4	6
> 2.050	76	82	60	40	18	23	4	5	6
> 2.900	79	82	70	30	18	23	3	5	7
> 5.150	82	82	80	20	18	23	2	5	8
> 7.100	79	79	80	20	21	22	2	6	8
> 7.600	76	75	80	20	25	21	2	7	8
> 9.500	79	75	90	10	25	21	1	7	9
> 12.40	76	71	90	10	29	20	1	8	9
> 21.90	79	71	100	0	29	20	0	8	10
> 32.35	76	68	100	0	32	19	0	9	10
> 35.10	74	64	100	0	36	18	0	10	10
> 37.00	71	61	100	0	39	17	0	11	10
> 42.30	68	57	100	0	43	16	0	12	10
> 46.40	66	54	100	0	46	15	0	13	10
> 50.35	63	50	100	0	50	14	0	14	10

Cys Depletion (%)	Accuracy (%)	Sensitivity (%)	Specificity (%)	False Positive (%)	False Negative (%)	Number of Substances Çorrect +	Number of Substances False +	Number of Substances False -	Number of Substances Çorrect -
> 55.25	61	46	100	0	54	13	0	15	10
> 59.35	58	43	100	0	57	12	0	16	10
> 73.50	55	39	100	0	61	11	0	17	10
> 86.05	53	36	100	0	64	10	0	18	10
> 88.90	50	32	100	0	68	9	0	19	10
> 91.05	47	29	100	0	71	8	0	20	10
> 93.20	45	25	100	0	75	7	0	21	10
> 95.00	42	21	100	0	79	6	0	22	10
> 95.80	39	18	100	0	82	5	0	23	10
> 97.40	37	14	100	0	86	4	0	24	10
> 99.20	34	11	100	0	89	3	0	25	10

Abbreviations: Correct + = correctly identified as positive; Correct - = correctly identified as negative; False + = incorrectly identified as positive; False - = incorrectly identified as negative; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; Cys = cysteine-containing peptide.

+ = sensitizer; - = nonsensitizer

Annex VIII

Reproducibility Analyses for the LLNA: DA
Using a Single Decision Criterion of SI ≥ 3.0 or SI ≥ 2.0

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1.0 LLNA: DA Test Method Reliability

An assessment of test method reliability (intralaboratory repeatability and intra- and interlaboratory reproducibility) is an essential element of any evaluation of the performance of an alternative test method (ICCVAM 2003). Repeatability refers to the closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period (ICCVAM 1997, 2003). Intralaboratory reproducibility refers to the extent to which qualified personnel within the same laboratory can replicate results using a specific test protocol at different times. Interlaboratory reproducibility refers to the extent to which different laboratories can replicate results using the same protocol and test substances, and indicates the extent to which a test method can be transferred successfully among laboratories. With regard to the murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content (referred to hereafter as the "LLNA: DA") test method, there are no known intralaboratory repeatability studies, which was also the situation with the traditional murine local lymph node assay (LLNA).

The LLNA: DA data were amenable to both intralaboratory and interlaboratory reproducibility analyses. The evaluation of a single decision criterion in Section 6.5 of this background review document (BRD) showed that stimulation index (SI) ≥ 1.8 produced the most optimum results (i.e., 93% accuracy and 0% false negative rate) among the alternate decision criteria evaluated. Thus Section 7.0 of this BRD provides an assessment of reproducibility for the decision criterion of SI ≥ 1.8 to identify potential sensitizers. Further, since SI ≥ 3.0 was used by the validation management team in the intralaboratory and interlaboratory validation studies, and SI ≥ 2.0 was previously evaluated as an optimum decision criterion in the March 2009 draft BRD reviewed by the independent scientific peer review Panel, this annex details additional reproducibility analyses for SI ≥ 3.0 and SI ≥ 2.0.

1.1 Intralaboratory Reproducibility (SI ≥ 3.0 or SI ≥ 2.0)

Idehara et al. (2008) evaluated the intralaboratory reproducibility of EC3 values (estimated concentration needed to produce an SI of three) for the LLNA: DA using two substances (isoeugenol and eugenol) that were each tested in three different experiments (**Table C-VIII-1**). The data indicate coefficients of variation (CVs) of 21% and 11% for isoeugenol and eugenol, respectively. The authors state that for both compounds the EC3 values appeared to be close and that for each test substance the SI values for the same concentration were fairly reproducible (Idehara et al. 2008). The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) also determined the intralaboratory reproducibility of EC2 values (estimated concentration needed to produce an SI of two) for the same set of data. The results for EC2 values with CV values of 35% and 20% for isoeugenol and eugenol, respectively, indicate slightly larger intralaboratory variability compared to EC3 value results.

1.2 Interlaboratory Reproducibility

Furthermore, data were submitted to NICEATM (Annex IV of this BRD) from a two-phased interlaboratory validation study on the LLNA: DA test method (Omori et al. 2008). In the first phase of the interlaboratory validation study, a blinded test of 12 substances was conducted in 10 laboratories. Three substances (i.e. 2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, and isopropanol) were tested in all 10 laboratories. The remaining nine substances were randomly assigned to subsets of three of the 10 laboratories **(Table C-VIII-2)**. In each laboratory, each substance was tested one time at three different concentrations. The dose levels for each substance were predetermined (i.e., the participating laboratories did not determine their own dose levels for testing). Nine substances are sensitizers and three substances are nonsensitizers according to traditional LLNA results. Six substances are ICCVAM-recommended LLNA performance standards

reference substances: cobalt chloride, 2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, isoeugenol, isopropanol, and methyl salicylate (ICCVAM 2009).

Table C-VIII-1 Intralaboratory Reproducibility of EC3 and EC2 Values Using the LLNA: DA[1]

Isoeugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
Vehicle (AOO)	1.00 ± 0.54	1.00 ± 0.54	1.00 ± 0.30
0.5	1.50 ± 0.54	-------	1.22 ± 0.13
1	2.28 ± 0.60	-------	2.77 ± 1.01
2.5	2.78 ± 0.17	3.11 ± 1.15	3.01 ± 0.98
5	3.39 ± 0.69	4.39 ± 1.25	-------
10	5.68 ± 1.19	6.77 ± 0.23	-------
EC3	3.40%	2.35%	2.46%
EC2	0.82%	1.37%	0.75%
Mean EC3: 2.74% ± 0.58% and 21% CV *Mean EC2: 0.98% ± 0.34% and 35% CV*			
Eugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
Vehicle (AOO)	1.00 ± 0.17	1.00 ± 0.17	1.00 ± 0.09
5	2.92 ± 1.00	2.80 ± 1.08	3.24 ± 0.70
10	7.35 ± 2.62	4.47 ± 0.98	4.79 ± 0.94
25	10.92 ± 3.63	5.62 ± 3.20	7.07 ± 0.44
EC3	5.09%	5.59%	4.50%
EC2	4.33%	3.59%	2.87%
Mean EC3: 5.06% ± 0.55% and 11% CV *Mean EC2: 3.60% ± 0.73% and 20% CV*			

Abbreviations: AOO = acetone: olive oil (4:1); CV = coefficient of variation; EC2 = estimated concentration needed to produce a stimulation index of two; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] Based on results discussed in Idehara et al. 2008; the number per group was not specified.

[2] Mean stimulation index value ± standard deviation.

Table C-VIII-2 Substances and Allocation for the First Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	Vehicle	Concentration Tested (%)			Laboratory									
					1	2	3	4	5	6	7	8	9	10
2,4-Dinitrochloro-benzene (+)	AOO	0.03	0.10	0.30	X	X	X	X	X	X	X	X	X	X
Hexyl cinnamic aldehyde (+)	AOO	5	10	25	X	X	X	X	X	X	X	X	X	X
Isopropanol (-)	AOO	10	25	50	X	X	X	X	X	X	X	X	X	X
Abietic acid (+)	AOO	5	10	25		X				X	X			
3-Aminophenol (+)	AOO	1	3	10	X		X					X		
Dimethyl isophthalate (-)	AOO	5	10	25	X		X				X			
Isoeugenol (+)	AOO	1	3	10				X	X				X	
Methyl salicylate (-)	AOO	5	10	25			X				X			X
Formaldehyde (+)	ACE	0.5	1.5	5.0	X	X			X					
Glutaraldehyde (+)	ACE	0.05	0.15	0.50	X	X			X					
Cobalt chloride[2] (+)	DMSO	0.3	1.0	3.0				X		X		X		
Nickel (II) sulfate hexahydrate (+)	DMSO	1	3	10				X		X		X		

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

The second phase of the interlaboratory validation study was designed to evaluate the reliability of the LLNA: DA for testing metallic salts using dimethyl sulfoxide (DMSO) as a vehicle since two metal salts dissolved in DMSO (cobalt chloride and nickel [II] sulfate hexahydrate) from the first phase of the interlaboratory validation study yielded inconsistent results. Five coded substances (two of the five substances were unique to the second phase of the interlaboratory validation study) were tested in seven laboratories (different from the 10 laboratories that performed the first interlaboratory validation study) **(Table C-VIII-3)**. One substance (i.e. hexyl cinnamic aldehyde) was tested in all seven laboratories. The remaining four substances (cobalt chloride, nickel [II] sulfate hexahydrate, lactic acid, and potassium dichromate) were randomly assigned to subsets of four of the seven laboratories. Each laboratory tested the substance one time at three different dose levels. Again, the dose levels for each substance were predetermined. Of the two substances not previously tested in the first phase of the interlaboratory validation study (lactic acid and potassium dichromate), one is a nonsensitizer and the other is a sensitizer according to traditional LLNA results, respectively. In addition, lactic acid is a reference substance included in performance standards recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM; ICCVAM 2009).

The LLNA: DA test results from the two-phased interlaboratory validation study are amenable to interlaboratory reproducibility analyses for three endpoints: sensitizer (positive) or nonsensitizer

(negative) classification (based on SI ≥ 3.0 and SI ≥ 2.0), and EC3 and EC2 values. Analyses of interlaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer based on SI ≥ 3.0 and SI ≥ 2.0) (**Sections 1.2.1** and **1.2.3,** respectively) and a CV analysis for the quantitative results (EC3 and EC2 values) (**Sections 1.2.2** and **1.2.4,** respectively).

Table C-VIII-3 Substances and Allocation for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	Vehicle	Concentration Tested (%)			Laboratory						
					11	12	13	14	15	16	17
Hexyl cinnamic aldehyde (+)	AOO	5	10	25	X	X	X	X	X	X	X
Cobalt chloride[2] (+)	DMSO	1	3	5	X		X	X			X
Lactic acid (-)	DMSO	5	10	25	X		X		X	X	
Nickel (II) sulfate hexahydrate (+)	DMSO	1	3	10	X	X		X		X	
Potassium dichromate (+)	DMSO	0.1	0.3	1.0	X	X			X		X

Abbreviations: AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional Murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

1.2.1 Interlaboratory Reproducibility – Qualitative Results (SI ≥ 3.0)

The qualitative (i.e., positive/negative) interlaboratory concordance analysis for the 12 substances that were tested during the first phase of the LLNA: DA interlaboratory validation study is shown in **Table C-VIII-4** using SI ≥ 3.0 as the decision criterion to distinguish sensitizers from nonsensitizers. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), eight substances tested in either three or 10 laboratories had consistent results leading to 100% (3/3 or 10/10) interlaboratory concordance for those substances. There were four discordant substances (formaldehyde, glutaraldehyde, cobalt chloride, and nickel [II] sulfate hexahydrate) for which interlaboratory concordance was 67% (2/3). One of the three laboratories that tested formaldehyde reported a maximum SI = 2.69 while the other two laboratories produced at least one SI ≥ 3.0. Similarly, one of the three laboratories that tested glutaraldehyde reported a maximum SI = 2.57 while the other two laboratories had at least one SI ≥ 3.0. Two of the three laboratories that tested cobalt chloride yielded an SI ≥ 3.0 at all three doses tested (0.3%, 1.0%, and 3.0%) and therefore classified the substance as a sensitizer similar to the traditional LLNA test method. Notably, the laboratory that did not generate an SI ≥ 3.0 did not test cobalt chloride at the highest dose and the middle dose yielded an SI = 2.66. One of the three laboratories that tested nickel (II) sulfate hexahydrate reported a maximum SI = 1.52, while the other two laboratories had at least two doses that yielded an SI ≥ 3.0. Since the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), there were no traditional LLNA concordance data for comparison with the LLNA: DA concordance data from the first phase of the interlaboratory validation study.

Table C-VIII-4 Qualitative Results for the First Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 3.0)

Substance Name[1]	Qualitative Results (Maximum SI)[2]										Concordance
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	
2,4-Dinitrochlorobenzene (+)	+ (11.97)	+ (9.23)	+ (9.96)	+ (8.53)	+ (7.86)	+ (15.14)	+ (13.18)	+ (12.60)	+ (10.89)	+ (4.71)	10/10
Hexyl cinnamic aldehyde (+)	+ (5.78)	+ (4.82)	+ (4.44)	+ (5.11)	+ (3.97)	+ (5.50)	+ (7.09)	+ (10.22)	+ (3.88)	+ (3.51)	10/10
Isopropanol (-)	- (1.54)	- (0.91)	- (1.01)	- (1.57)	- (0.76)	- (1.97)	- (1.45)	- (1.21)	- (0.70)	- (1.25)	10/10
Abietic acid (+)		+ (4.64)				+ (7.96)	+ (3.98)				3/3
3-Aminophenol (+)	- (2.83)		- (1.76)					- (2.38)			3/3
Dimethyl isophthalate (-)	- (1.34)		- (1.29)				- (1.26)				3/3
Isoeugenol (+)				+ (6.11)	+ (5.54)				+ (7.09)		3/3
Methyl salicylate (-)			- (1.55)				- (1.77)			- (0.83)	3/3
Formaldehyde (+)	**+ (4.84)**	**+ (3.18)**			**- (2.69)**						**2/3**
Glutaraldehyde (+)	**+ (5.00)**	**+ (3.39)**			**- (2.57)**						**2/3**
Cobalt chloride[3] (+)				**-[4] (2.66)**		**+ (20.55)**		**+ (8.07)**			**2/3**
Nickel (II) sulfate hexahydrate (+)				**-[5] (1.52)**		**+ (11.78)**		**+[5] (3.49)**			**2/3**

Bolded substances did not achieve 100% interlaboratory concordance.

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] (+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests. Highest stimulation index value for each test is shown in parentheses.

[3] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

[4] Data not reported for the highest dose (3%), only for 0.3% and 1%.

[5] Insufficient dose response.

The qualitative (positive/negative) interlaboratory concordance analysis for the five substances that were tested during the second phase of the LLNA: DA interlaboratory validation study is shown in **Table C-VIII-5** using SI ≥ 3.0 as the decision criterion to distinguish sensitizers from nonsensitizers. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), four substances (hexyl cinnamic aldehyde, lactic acid, nickel [II] sulfate hexahydrate, and potassium dichromate) tested in either four or seven laboratories had consistent results leading to 100% (4/4 or 7/7) interlaboratory concordance for those substances. There was one discordant substance (cobalt chloride) for which interlaboratory concordance was 50% (2/4). Two of the four laboratories that tested cobalt chloride reported a maximum SI = 2.01 and 2.54, respectively, while the other two laboratories had at least two doses that yielded an SI ≥ 3.0. As was discussed previously, cobalt chloride was also discordant among the laboratories that tested the substance in the first phase of the interlaboratory validation study and interlaboratory concordance was 67% (2/3). Notably, different doses of cobalt chloride were tested in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study. Furthermore, as mentioned previously, the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), and therefore there were no traditional LLNA concordance data for comparison with the LLNA: DA concordance data from the second phase of the interlaboratory validation study.

Table C-VIII-5 Qualitative Results for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 3.0)

Substance Name[1]	Qualitative Results (Maximum SI)[2]							Concordance
	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17	
Hexyl cinnamic aldehyde (+)	+ (4.47)	+ (5.71)	+ (5.41)	+ (7.60)	+ (3.92)	+ (8.42)	+ (6.45)	7/7
Cobalt chloride[3] (+)	**- (2.01)**		**- (2.54)**	**+ (4.25)**			**+ (5.06)**	**2/4**
Lactic acid (-)	- (0.93)		- (0.99)		- (0.97)	- (0.91)		4/4
Nickel (II) sulfate hexahydrate (+)	- (0.79)	- (1.24)		- (2.13)		- (1.56)		4/4
Potassium dichromate (+)	+ (4.78)	+ (4.08)			+ (6.01)		+ (6.37)	4/4

Boldface type indicates substances that did not achieve 100% interlaboratory concordance.

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP Content; SI = stimulation index.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] (+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests. Highest stimulation index value for each test is shown in parentheses.

[3] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

1.2.2 Interlaboratory Reproducibility – EC3 Values

The available quantitative (i.e., EC3 value) data for interlaboratory reproducibility analysis were obtained from the LLNA: DA tests that yielded positive results (SI ≥ 3.0) during the first and second phase of the LLNA: DA interlaboratory validation study. The method for calculating EC3 values for the positive results was based on the method of linear interpolation reported by Gerberick et al. (2004) according to the equation:

$$EC3 = c + \left[\frac{(3-d)}{(b-d)}\right] \times (a-c)$$

where the data points lying immediately above and below the SI = 3.0 on the dose response curve have the coordinates of (a, b) and (c, d), respectively (Gerberick et al. 2004). For substances for which the lowest concentration tested resulted in an SI ≥ 3.0, an EC3 value was extrapolated according to the equation:

$$EC3_{ex} = 2^{\left\{\log_2(c)+\frac{(3-d)}{(b-d)}\times\left[\log_2(a)-\log_2(c)\right]\right\}}$$

where the point with the higher SI is denoted with the coordinates of (a, b) and the point with the lower SI is denoted (c, d) (Gerberick et al. 2004).

The EC3 values from each laboratory were used to calculate CV values for each substance. The resulting values for the first and second phase of the interlaboratory validation study are shown in **Tables C-VIII-6** and **C-VIII-7**, respectively. In the first phase of the interlaboratory validation study, CV values ranged from 4% (abietic acid) to 84% (glutaraldehyde) and the mean CV was 48% (**Table C-VIII-6**). Notably, although nickel (II) sulfate hexahydrate was a sensitizer in two of three laboratories, a CV could not be determined because one of the two laboratories that yielded a positive test demonstrated an insufficient dose response (i.e., an inverse dose response curve) from which to calculate an EC3 value. In the second phase of the interlaboratory validation study, CV values ranged from 32% (cobalt chloride) to 71% (potassium dichromate) and the mean CV was 45% (**Table C-VIII-7**).

The ICCVAM-recommended LLNA performance standards (ICCVAM 2009) indicate that interlaboratory reproducibility should be evaluated with at least two sensitizing chemicals with well-characterized activity in the traditional LLNA. Acceptable reproducibility is attained when each laboratory obtains ECt values (estimated concentration needed to produce an SI of a specified threshold) within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20% for hexyl cinnamic aldehyde (ICCVAM 2009). In the first phase of the interlaboratory validation study, four laboratories reported EC3 values outside the range indicated for 2,4-dinitrochlorobenzene; one laboratory obtained an EC3 value that was lower than the specified acceptance range (0.025%) and three laboratories obtained EC3 values that were higher than the specified acceptance range (0.1%) (**Table C-VIII-6**). For hexyl cinnamic aldehyde, all the laboratories obtained an EC3 value within the acceptance range (5% to 20%). In the second phase of the interlaboratory validation study, only hexyl cinnamic aldehyde was tested and all seven laboratories obtained EC3 values that were within the acceptance range indicated (**Table C-VIII-7**).

Table C-VIII-6EC3 Values from the First Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	EC3 (%)										Mean EC3 (%) ± SD	CV (%)
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10		
2,4-Dinitrochlorobenzene (+)	**0.034 (11.97)**	**0.109 (9.23)**	**0.056 (9.96)**	**0.031 (8.53)**	**0.129 (7.86)**	**0.042 (15.14)**	**0.016 (13.18)**	**0.095 (12.60)**	**0.040 (10.89)**	**0.169 (4.71)**	**0.072 ± 0.051**	**70**
Hexyl cinnamic aldehyde (+)	**9.983 (5.78)**	**12.412 (4.82)**	**14.90 (4.44)**	**9.340 (5.11)**	**18.131 (3.97)**	**13.130 (5.50)**	**7.706 (7.09)**	**7.924 (10.22)**	**17.070 (3.88)**	**15.235 (3.51)**	**12.583 ± 3.748**	**30**
Isopropanol (-)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Abietic acid (+)		8.196				7.544	7.676				7.805 ± 0.345	4
3-Aminophenol (+)	NA		NA					NA			NA	NA
Dimethyl isophthalate (-)	NA		NA				NA				NA	NA
Isoeugenol (+)				1.112	5.983				2.300		3.131 ± 2.540	81
Methyl salicylate (-)			NA				NA			NA	NA	NA
Formaldehyde (+)	1.747	1.480			NA						1.614 ± 0.189	12
Glutaraldehyde (+)	0.110	0.435			NA						0.272 ± 0.230	84
Cobalt chloride[2] (+)				NA[3]		0.063		0.137			0.100 ± 0.053	53
Nickel (II) sulfate hexahydrate (+)				NA		0.469		IDR			0.469 ± NA	NA

Note: Bolded text indicates substances that are ICCVAM-recommended murine local lymph node assay (LLNA) performance standards reference substances for evaluating interlaboratory reproducibility (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For both 2,4-dinitrochlorobenzene and hexyl cinnamic aldehyde, the highest SI values achieved are from the highest dose tested (0.30% for 2,4-dinitrochlorobenzene and 25% for hexyl cinnamic aldehyde). Shading shows EC3 values (estimated concentration needed to produce an SI of three) that are outside of the acceptable range indicated in the ICCVAM-recommended LLNA performance standards: 5 - 20% for hexyl cinnamic aldehyde and 0.025 - 0.1% for 2,4-dinitrochlorobenzene.

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; IDR = insufficient dose response; NA = not applicable; SD = standard deviation.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and second phase (1%, 3%, and 10%) of the interlaboratory validation study.

[3] Data not reported for the highest dose (3%), only for 0.3% and 1%.

Table C-VIII-7 EC3 Values from the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	EC3 (%)							Mean EC3 (%) ± SD	CV (%)
	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17		
Hexyl cinnamic aldehyde (+)	**9.127 (4.47)**	**8.764 (5.71)**	**7.590 (5.41)**	**7.938 (7.60)**	**15.184 (3.92)**	**6.230 (8.42)**	**7.542 (6.45)**	**8.911 ± 2.920**	**33**
Cobalt chloride[2] (+)	NA		NA	1.761			1.109	1.435 ± 0.461	32
Lactic acid (-)	NA		NA		NA	NA		NA	NA
Nickel (II) sulfate hexahydrate (+)	NA	NA		NA		NA		NA	NA
Potassium dichromate (+)	0.509	0.485			0.156		0.086	0.309 ± 0.219	71

Bolded text indicates a substance that is an ICCVAM-recommended murine local lymph node assay performance standards reference substance for evaluating interlaboratory reproducibility (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For hexyl cinnamic aldehyde, the highest SI values achieved are from the highest dose tested (25%). None of the EC3 values are outside of the acceptable range indicated in the ICCVAM-recommended LLNA performance standards (5 - 20% for hexyl cinnamic aldehyde).

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; NA = not applicable; SD = standard deviation.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

The interlaboratory CV values for both the first and second phases of the interlaboratory validation study for the LLNA: DA EC3 values were higher than that for the traditional LLNA EC3 values. The analysis of interlaboratory variation of EC3 values for the traditional LLNA reported CV values of 6.8 to 83.7% for five substances tested in five laboratories (**Table C-VIII-8**; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: DA (hexyl cinnamic aldehyde, 2,4-dinitrochlorobenzene, and isoeugenol). All interlaboratory CV values for the LLNA: DA were greater than that for the traditional LLNA. The CV of 70% for 2,4-dinitrochlorobenzene was greater than the two CV values of 37.4% and 27.2%, calculated from five values each, reported by ICCVAM (1999). The CV values of 30% and 33% for hexyl cinnamic aldehyde tested in the first and second phase of the LLNA: DA interlaboratory validation study, respectively, were both greater than the 6.8% reported by ICCVAM (1999). The CV of 81% for isoeugenol tested in the LLNA: DA was greater than the 41.2% reported by ICCVAM (1999).

Table C-VIII-8 Interlaboratory Reproducibility of the EC3 Values for Substances Tested in the Traditional LLNA[1]

Substance Name	EC3 (%)					CV (%)
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	
2,4-Dinitrochlorobenzene	0.3	0.5	0.6	0.9	0.6	37.4
	0.5	0.6	0.4	0.6	0.3	27.2
Hexyl cinnamic aldehyde	7.9	7.6	8.4	7.0	8.1	6.8
Isoeugenol	1.3	3.3	1.8	3.1	1.6	41.2
Eugenol	5.8	14.5	8.9	13.8	6.0	42.5
Sodium lauryl sulfate	13.4	4.4	1.5	17.1	4.0	83.7

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA = murine local lymph node assay.

[1] From ICCVAM 1999 report.

1.2.3 Interlaboratory Reproducibility – Qualitative Results (SI ≥ 2.0)

The qualitative (positive/negative) interlaboratory concordance analysis for the 12 substances that were tested during the first phase of the LLNA: DA interlaboratory validation study is shown in **Table C-VIII-9** for SI ≥ 2.0. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), ten substances tested in either three or 10 laboratories had consistent results leading to 100% (3/3 or 10/10) interlaboratory concordance for those substances. There were two discordant substances (3-aminophenol and nickel [II] sulfate hexahydrate) for which interlaboratory concordance was 67% (2/3). Two of the three laboratories that tested 3-aminophenol reported SI ≥ 2.0, at least at the highest dose tested (SI = 2.83 and 2.38, respectively) but one lab did not achieve SI ≥ 2.0 at any dose tested (Annex IV of this BRD). One of the three laboratories that tested nickel (II) sulfate hexahydrate reported a maximum SI = 1.52, while the other two laboratories produced SI ≥ 2.0 at all three doses tested (Annex IV of this BRD). Since the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), there were no traditional LLNA concordance data for comparison with the LLNA: DA concordance data from the first phase of the interlaboratory validation study.

Table C-VIII-9 Qualitative Results for the First Phase of the Interlaboratory Validation Studies for the LLNA: DA (SI ≥ 2.0)

Substance Name[1]	Qualitative Results (Maximum SI)[2]										Concordance
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	
2,4-Dinitrochlorobenzene (+)	+ (11.97)	+ (9.23)	+ (9.96)	+ (8.53)	+ (7.86)	+ (15.14)	+ (13.18)	+ (12.60)	+ (10.89)	+ (4.71)	10/10
Hexyl cinnamic aldehyde (+)	+ (5.78)	+ (4.82)	+ (4.44)	+ (5.11)	+ (3.97)	+ (5.50)	+ (7.09)	+ (10.22)	+ (3.88)	+ (3.51)	10/10
Isopropanol (-)	- (1.54)	- (0.91)	- (1.01)	- (1.57)	- (0.76)	- (1.97)	- (1.45)	- (1.21)	- (0.70)	- (1.25)	10/10
Abietic acid (+)		+ (4.64)				+ (7.96)	+ (3.98)				3/3
3-Aminophenol (+)	**+ (2.83)**		**- (1.76)**					**+ (2.38)**			**2/3**
Dimethyl isophthalate (-)	- (1.34)		- (1.29)				- (1.26)				3/3
Isoeugenol (+)				+ (6.11)	+ (5.54)				+ (7.09)		3/3
Methyl salicylate (-)			- (1.55)				- (1.77)			- (0.83)	3/3
Formaldehyde (+)	+ (4.84)	+ (3.18)			+ (2.69)						3/3
Glutaraldehyde (+)	+ (5.00)	+ (3.39)			+ (2.57)						3/3
Cobalt chloride[3] (+)				+[4] (2.66)		+ (20.55)		+ (8.07)			3/3
Nickel (II) sulfate hexahydrate (+)				**-[5] (1.52)**		**+ (11.78)**		**+[5] (3.49)**			**2/3**

Boldface text indicates substances did not achieve 100% interlaboratory concordance.

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] (+) indicates sensitizer result and (-) indicates nonsensitizer result in the LLNA: DA test. Highest stimulation index value for each test is shown in parentheses.

[3] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

[4] Data not reported for the highest dose (3%), only for 0.3% and 1%.

[5] Insufficient dose response.

The qualitative (positive/negative) interlaboratory concordance analysis for the five substances that were tested during the second phase of the LLNA: DA interlaboratory validation study is shown in **Table C-VIII-10**. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), four substances (hexyl cinnamic aldehyde, cobalt chloride, lactic acid, and potassium dichromate) tested in either four or seven laboratories had consistent results leading to 100% (4/4 or 7/7) interlaboratory concordance for those substances. There was one discordant substance (nickel [II] sulfate hexahydrate) for which interlaboratory concordance was 75% (3/4). Three of the four laboratories that tested nickel (II) sulfate hexahydrate did not report a maximum SI ≥ 2.0, while the other laboratory produced an SI ≥ 2.0 at the highest dose tested. As was discussed previously, nickel (II) sulfate hexahydrate was also discordant among the laboratories that tested the substance in the first phase of the interlaboratory validation study and interlaboratory concordance was 67% (2/3). Notably, when analyzing the dose response curves for the seven tests performed for nickel (II) sulfate hexahydrate in the two-phased interlaboratory validation study, only one study demonstrated a sufficient dose response (i.e., a parallel increase in SI relative to increase in concentration). Furthermore, as mentioned previously, the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), and therefore there were no traditional LLNA concordance data for comparison with the LLNA: DA concordance data from the second phase of the interlaboratory validation study.

Table C-VIII-10 Qualitative Results for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 2.0)

Substance Name[1]	Qualitative Results (Maximum SI)[2]							Concordance
	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17	
Hexyl cinnamic aldehyde (+)	+ (4.47)	+ (5.71)	+ (5.41)	+ (7.60)	+ (3.92)	+ (8.42)	+ (6.45)	7/7
Cobalt chloride[3] (+)	+ (2.01)		+ (2.54)	+ (4.25)			+ (5.06)	4/4
Lactic acid (-)	- (0.93)		- (0.99)		- (0.97)	- (0.91)		4/4
Nickel (II) sulfate hexahydrate (+)	**- (0.79)**	**- (1.24)**		**+ (2.13)**		**- (1.56)**		**3/4**
Potassium dichromate (+)	+ (4.78)	+ (4.08)			+ (6.01)		+ (6.37)	4/4

Boldface text indicates substance that did not achieve 100% interlaboratory concordance.

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] (+) indicates sensitizer result and (-) indicates nonsensitizer result in the LLNA: DA test. Highest stimulation index value for each test is shown in parentheses.

[3] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) interlaboratory validation studies.

1.2.4 Interlaboratory Reproducibility – EC2 Values

The available quantitative (i.e., EC2 value) data for interlaboratory reproducibility analysis were obtained from the LLNA: DA tests that yielded positive results (i.e., SI ≥ 2.0) during the first and second phase of the LLNA: DA interlaboratory validation study. The equation used for calculating EC2 values for the positive results was modified based on the method of linear interpolation reported by Gerberick et al. (2004) for the EC3 value:

$$EC2 = c + \left[\frac{(2-d)}{(b-d)}\right] \times (a-c)$$

where the data points lying immediately above and below the SI = 2.0 on the dose response curve have the coordinates of (a, b) and (c, d), respectively (Gerberick et al. 2004). For substances for which the lowest concentration tested resulted in an SI ≥ 2.0, an EC2 value was extrapolated according to the equation:

$$EC2_{ex} = 2^{\left\{\log_2(c)+\frac{(2-d)}{(b-d)}\times\left[\log_2(a)-\log_2(c)\right]\right\}}$$

where the point with the higher SI is denoted with the coordinates of (a, b) and the point with the lower SI is denoted (c, d) (Gerberick et al. 2004).

The EC2 values from each laboratory were used to calculate CV values for each substance. The resulting values for the first and second phase of the interlaboratory validation study are shown in **Tables C-VIII-11** and **C-VIII-12**, respectively. In the first phase of the interlaboratory validation study, CV values ranged from 14% (abietic acid) to 134% (isoeugenol) and the mean CV was 70% (**Table C-VIII-11**). In the second phase of the interlaboratory validation study, CV values ranged from 16% (hexyl cinnamic aldehyde) to 100% (cobalt chloride) and the mean CV was 57% (**Table C-VIII-12**).

The ICCVAM-recommended LLNA performance standards indicate that interlaboratory reproducibility should be evaluated with at least two sensitizing chemicals with well-characterized activity in the traditional LLNA (ICCVAM 2009). Acceptable reproducibility is attained when each laboratory obtains ECt values (estimated concentration needed to produce an SI of a specific threshold) within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20% for hexyl cinnamic aldehyde (ICCVAM 2009). In the first phase of the interlaboratory validation study, seven laboratories reported EC2 values outside the range indicated for 2,4-dinitrochlorobenzene; all seven laboratories obtained EC2 values that were lower than the specified acceptance range (0.025%) (**Table C-VIII-11**). For hexyl cinnamic aldehyde, all the laboratories obtained an EC2 value within the acceptance range (5% to 20%). In the second phase of the interlaboratory validation study, only hexyl cinnamic aldehyde was tested and two of the seven laboratories obtained EC2 values that were below the acceptance range indicated (**Table C-VIII-12**).

Table C-VIII-11 EC2 Values from the First Phase Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	EC2 (%)										Mean EC2 (%) ± SD	CV (%)
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10		
2,4-Dinitrochlorobenzene (+)	**0.020 (11.97)**	**0.023 (9.23)**	**0.026 (9.96)**	**0.016 (8.53)**	**0.091 (7.86)**	**0.016 (15.14)**	**0.007 (13.18)**	**0.013 (12.60)**	**0.019 (10.89)**	**0.093 (4.71)**	**0.032 ± 0.032**	**98**
Hexyl cinnamic aldehyde (+)	**6.962 (5.78)**	**7.461 (4.82)**	**8.404 (4.44)**	**6.460 (5.11)**	**11.057 (3.97)**	**7.463 (5.50)**	**5.850 (7.09)**	**6.140 (10.22)**	**9.191 (3.88)**	**7.256 (3.51)**	**7.624 ± 1.570**	**21**
Isopropanol (-)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Abietic acid (+)		4.760				5.393	6.333				5.495 ± 0.791	14
3-Aminophenol (+)	1.877		NA					3.179			2.528 ± 0.921	36
Dimethyl isophthalate (-)	NA		NA				NA				NA	NA
Isoeugenol (+)				0.407	4.399				0.375		1.727 ± 2.314	134
Methyl salicylate (-)			NA				NA			NA	NA	NA
Formaldehyde (+)	0.262	0.729			2.019						1.003 ± 0.910	91
Glutaraldehyde (+)	0.072	0.268			0.118						0.153 ± 0.103	67
Cobalt chloride[2] (+)				0.283[3]		0.032		0.079			0.131 ± 0.134	102
Nickel (II) sulfate hexahydrate (+)				IDR		0.235		IDR			0.235 ± NA	NA

Bolded text indicates substances that are ICCVAM-recommended murine local lymph node assay (LLNA) performance standards reference substances for evaluating interlaboratory reproducibility (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For both 2,4-dinitrochlorobenzene and hexyl cinnamic aldehyde, the highest SI values achieved were from the highest dose tested (0.30% for 2,4-dinitrochlorobenzene and 25% for hexyl cinnamic aldehyde). Shading shows EC2 values that are outside of the acceptable range indicated by the ICCVAM-recommended LLNA performance standards: 5 - 20% for hexyl cinnamic aldehyde and 0.025 - 0.1% for 2,4-dinitrochlorobenzene.

Abbreviations: CV = coefficient of variation; EC2 = estimated concentration needed to produce a stimulation index of two; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; IDR = insufficient dose response; NA = not applicable; SD = standard deviation.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) interlaboratory validation studies.

[3] Data not reported for the highest dose (3%), only for 0.3% and 1%.

Table C-VIII-12 EC2 Values from the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	EC2 (%)							Mean EC2 (%) ± SD	CV (%)
	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17		
Hexyl cinnamic aldehyde (+)	**6.348 (4.47)**	**5.983 (5.71)**	**5.954 (5.41)**	**4.849 (7.60)**	**7.451 (3.92)**	**4.662 (8.42)**	**6.024 (6.45)**	**5.896 ± 0.937**	**16**
Cobalt chloride[2] (+)	4.929		1.875	0.821			0.461	2.021 ± 2.029	100
Lactic acid (-)	NA		NA		NA	NA		NA	NA
Nickel (II) sulfate hexahydrate (+)	NA	NA		NA		8.404		8.404 ± NA	NA
Potassium dichromate (+)	0.159	0.128			0.055		0.047	0.097 ± 0.055	56

Bolded text indicates a substance that is an ICCVAM-recommended murine local lymph node assay (LLNA) performance standards reference substance for evaluating interlaboratory reproducibility (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For hexyl cinnamic aldehyde, the highest SI values achieved were from the highest dose tested (25%). Two of the EC2 values are outside of the acceptable range indicated by the ICCVAM-recommended LLNA performance standards (5 - 20% for hexyl cinnamic aldehyde), indicated by shading.

Abbreviations: CV = coefficient of variation; EC2 = estimated concentration needed to produce a stimulation index of two; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; NA = not applicable; SD = standard deviation.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

The interlaboratory CV values for both the first and second phases of the interlaboratory validation study for the LLNA: DA EC2 values were higher than that for the traditional LLNA EC3 values. The analysis of interlaboratory variation of EC3 values for the traditional LLNA reported CV values of 6.8 to 83.7% for five substances tested in five laboratories (**Table C-VIII-8**; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: DA (hexyl cinnamic aldehyde, 2,4-dinitrochlorobenzene, and isoeugenol). All interlaboratory CV values for LLNA: DA EC2 values were greater than that for the traditional LLNA. The CV of 98% for 2,4-dinitrochlorobenzene was greater than the two CV values of 37.4% and 27.2% (which were calculated from five values each), reported by ICCVAM (1999). The CV of 21% and 16% for hexyl cinnamic aldehyde tested in the first and second phase of the LLNA: DA interlaboratory validation study, respectively, were both greater than the 6.8% reported by ICCVAM (1999). The CV of 134% for isoeugenol tested in the LLNA: DA was greater than the 41.2% reported by ICCVAM (1999).

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Appendix D

Independent Scientific Peer Review Panel Assessment

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Appendix D1

Summary Minutes from the Independent Scientific Peer Review Panel Meeting on March 4-6, 2008

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Summary Minutes

Independent Scientific Peer Review Panel Meeting

Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products

Consumer Product Safety Commission (CPSC), Headquarters
Bethesda, MD
March 4 – 6, 2008
8:30 a.m. – 5:30 p.m.

Peer Review Panel Members:

Michael Luster, Ph.D. (Peer Review Panel Chair)	Senior Consultant to the NIOSH Health Effects Laboratory, Morgantown, WV, U.S.
Nathalie Alépée, Ph.D.	Associate Research Fellow, Pfizer PDRD MCT Laboratory, France
Anne Marie Api, Ph.D.	Vice President, Human Health Sciences, Research Institute for Fragrance Materials, Woodcliff Lake, NJ, U.S.
Nancy Flournoy, M.S., Ph.D.	Professor and Chair, Dept. of Mathematics and Statistics, University of Missouri-Columbia, Columbia, MO, U.S.
Thomas Gebel, Ph.D.	Regulatory Toxicologist, Federal Institute for Occupational Safety and Health, Dortmund, Germany
Kim Headrick, B. Admin., B.Sc.	International Harmonization Senior Policy Advisor, Health Canada, Ottawa, Ontario, Canada
Dagmar Jírová, M.D., Ph.D.	Toxicologist, Research Manager, Head of Reference Center for Cosmetics, Head of Reference Laboratory for Experimental Immunotoxicology, National Institute of Public Health, Czech Republic
David Lovell, Ph.D	Reader in Medical Statistics, Postgraduate Medical School, University of Surrey, Guildford, Surrey, U.K.
Howard Maibach, M.D.	Professor, Dept. of Dermatology, University of California-San Francisco, San Francisco, CA, U.S.

Peer Review Panel Members:

James McDougal, Ph.D.	Professor and Director of Toxicology Research, Dept. of Pharmacology and Toxicology, Boonshoft School of Medicine, Wright State University, Dayton, OH, U.S.
Michael Olson, Ph.D.	Director of Occupational Toxicology, Corporate Environment Health and Safety, GlaxoSmithKline, RTP, NC, U.S.
Raymond Pieters, Ph.D.	Associate Professor, Immunotoxicology Group Leader, Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands
Jean Regal, Ph.D.	Professor, Dept. of Pharmacology, University of Minnesota Medical School, Duluth, MN, U.S.
Peter Theran, V.M.D.	Massachusetts Society for the Prevention of Cruelty to Animals, Novato, CA, U.S.
Stephen Ullrich, Ph.D.	Dallas/Ft. Worth Living Legends Professor & Professor of Immunology, Graduate School of Biomedical Science, University of Texas M.D. Anderson Cancer Center, Houston, TX, U.S.
Michael Woolhiser, Ph.D.	Technical Leader - Immunotoxicology, Toxicology and Environmental Research and Consulting Immunology, Dow Chemical, Midland, MI, U.S.
Takahiko Yoshida, M.D., Ph.D.	Professor, Dept. of Health Science, Asahikawa Medical College, Hokkaido, Japan

ICCVAM and ICCVAM IWG Members:

Paul Brown, Ph.D.	FDA, Silver Spring, MD, U.S.
Ruth Barratt, Ph.D., D.V.M.	FDA, Rockville, MD, U.S.
Karen Hamernik, Ph.D.	EPA, Washington, DC, U.S.
Masih Hashim, Ph.D.	EPA, Washington, DC, U.S.
Abigail Jacobs, Ph.D. (IWG Co-Chair)	FDA, Silver Spring, MD, U.S.
Kristina Hatlelid, Ph.D.	CPSC, Bethesda, MD, U.S.
Joanna Matheson, Ph.D. (IWG Co-Chair)	CPSC, Bethesda, MD, U.S.
Tim McMahon, Ph.D.	EPA, Washington, DC, U.S.

ICCVAM and ICCVAM IWG Members:

Amy Rispin, Ph.D.	EPA, Washington, DC, U.S.
William Stokes, D.V.M., DACLAM	NIEHS, RTP, NC, U.S.
Raymond Tice, Ph.D.	NIEHS, RTP, NC, U.S.
Ron Ward, Ph.D.	EPA, Washington, DC, U.S.
Marilyn Wind, Ph.D. (ICCVAM Chair)	CPSC, Bethesda, MD, U.S.
Jiaqin Yao, Ph.D.	FDA, Silver Spring, MD, U.S.

ECVAM Observer:

David Basketter, Ph.D.	DABMEB Consultancy Ltd., Bedfordshire, U.K.

Invited Experts:

George DeGeorge, Ph.D., DABT	MB Research Laboratories, Spinnerstown, PA, U.S.
Kenji Idehara, Ph.D.	Daicel Chemical Industries, Hyogo, Japan
Masahiro Takeyoshi, Ph.D.	Chemicals Evaluation and Research Institute, Saitama, Japan

Public Attendees:

Odette Alexander	Syngenta Crop Protection, Inc., Greensboro, NC, U.S.
Nancy Beck, Ph.D.	PCRM, Washington, DC, U.S.
Ann Blacker, Ph.D.	Bayer CropScience, RTP, NC, U.S.
Stuart Cagan, Ph.D.	Shell Oil Company, Houston, TX, U.S.
Joan Chapdelaine, Ph.D.	Calvert Laboratories, Inc., Olyphant, PA, U.S.
Adriana Doi, Ph.D.	BASF Corporation, RTP, NC, U.S.
Carol Eisenmann, Ph.D.	Personal Care Products Council, Washington, DC, U.S.
Charles Hastings, Ph.D.	BASF Corporation, RTP, NC, U.S.
Kailash Gupta, D.V.M., Ph.D.	Retired CPSC, Bethesda, MD, U.S.
John Lyssikatos	Hill Top Research, Miamiville, OH, U.S.
Laurence Musset, Ph.D.	OECD, Paris, France
Carol O'Neil	NuPathe, Conshohocken, PA, U.S.

Public Attendees:

Kui Lea Park, Ph.D.	National Institute of Toxicological Research, KFDA, Seoul, Korea
Rafael Rivas	AFRRI/USHUS, Bethesda, MD, U.S.
Terri Sebree	NuPathe, Conshohocken, PA, U.S.
Libby Sommer	EPA, Washington, DC, U.S.
Merrill Tisdel	Syngenta Crop Protection Inc., Greensboro, NC, U.S.
Jeffrey Toy, Ph.D.	FDA, Rockville, MD, U.S.

NICEATM:

William Stokes, D.V.M., DACLAM	Director
Raymond Tice, Ph.D.	Deputy Director
Debbie McCarley	Special Assistant to the Director

Support Contract Staff— Integrated Laboratory Systems, Inc. (ILS)

David Allen, Ph.D.	Michael Paris
Thomas Burns, M.S.	Eleni Salicru, Ph.D.
Linda Litchfield	Judy Strickland, Ph.D., DABT
Douglas Winters, M.S.	

Abbreviations:

AFFRI = Armed Forces Radiobiology Research Institute

CPSC = U.S. Consumer Product Safety Commission

ECVAM = European Centre for the Validation of Alternative Methods

EPA = U.S. Environmental Protection Agency

FDA = U.S. Food and Drug Administration

ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods

ILS = Integrated Laboratory Systems

IWG = Immunotoxicology Working Group

KFDA = Korea Food and Drug Administration

NICEATM = National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

NIEHS = National Institute of Environmental Health Sciences

NIOSH = National Institute of Occupational Safety and Health

OECD = Organisation for Economic Co-operation and Development

PCRM = Physicians Committee for Responsible Medicine

USDA = U.S. Department of Agriculture

USHUS = Uniformed Services University of the Health Sciences

TUESDAY, MARCH 4, 2008

Call to Order and Introductions—

Dr. Michael Luster (Peer Review Panel Chair) called the meeting to order at 8:30 a.m. and introduced himself. He then asked all Peer Review Panel (hereafter Panel) members to introduce themselves and to state their name and affiliation for the record. He then asked all the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) staff, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) members, the ICCVAM Immunotoxicity Working Group (IWG) members, the European Centre for the Validation of Alternative Methods (ECVAM) observer, and members of the public to also introduce themselves. Dr. Luster stated that there would be opportunity for public comments during each of the seven local lymph node assay (LLNA)-related topics. He asked that all those interested in making a comment register at the registration table and provide a written copy of their comments, if available, to NICEATM staff. Dr. Luster emphasized that the comments would be limited to seven minutes per individual and that, while an individual would be welcome to make comments during each commenting period, repeating the same comments at each comment period would be inappropriate. He further stated that the meeting was being recorded and that Panel members should speak directly their microphone. Finally, Dr. Luster noted that if the Panel finished early with the assigned topics on the agenda for that day, they would proceed to the next day's topics if time permitted.

Welcome from the ICCVAM Chair—

Dr. Marilyn Wind, U.S. Consumer Product Safety Commission (CPSC) and Chair of ICCVAM, welcomed everyone to CPSC and to the Panel meeting. Dr. Wind stressed the importance of this Panel's efforts especially considering recent reports that allergies and asthma have increased markedly over the past number of years and that contact dermatitis is the most common occupational illness in the United States. Dr. Wind thanked the Panel members for giving their expertise, time, and effort and acknowledged their important role to the ICCVAM test method evaluation process. Dr. Wind also emphasized the important role of the public and their comments in this process.

Welcome from the Director of NICEATM, and Conflict of Interest Statements—

Dr. William Stokes, Director of NICEATM, stated the Panel meeting was being convened as a National Institutes of Health (NIH) special emphasis panel and was being held in accordance with the Federal Advisory Committee Act regulations. As such, Dr. Stokes indicated that he would serve as the Designated Federal Official for this public meeting. He reminded the Panel that they had signed a conflict-of-interest statement when they were selected for the Panel, in which they identified any potential conflicts of interest. He then read this statement to provide another opportunity for members of the Panel to identify any conflicts not previously declared. Dr. Luster asked the Panel members to declare any direct or indirect conflicts based on Dr. Stokes statements and to recuse themselves from discussion and voting on any aspect of the meeting where there might be a conflict. None of the Panel members declared a conflict of interest.

Overview of the ICCVAM Test Method Evaluation Process

Dr. Stokes provided an overview of the ICCVAM test method evaluation process. He stated that the Panel was made up of 19 different scientists from eight different countries (Canada, Czech Republic, France, Germany, Japan, The Netherlands, United Kingdom, and the United States). Dr. Stokes thanked the Panel members for the significant amount of time and effort that they had devoted to prepare for and attend the meeting. He explained that the purpose of the Panel was to assist ICCVAM by carrying out an independent scientific peer review of the information provided on a series of proposed new versions of the LLNA and some expanded applications of the assay. Dr. Stokes

mentioned that the original LLNA peer review panel in 1998 considered the LLNA a valid substitute for the guinea pig-based test in most testing situations, but not all. He mentioned that three Panel members from the 1998 review are also on the current Panel (i.e., Drs. Howard Maibach, Jean Regal, and Stephen Ullrich). Dr. Stokes also reviewed the nomination that was received from CPSC in January 2007,[1] which provides the basis for the current evaluation.

Dr. Stokes then identified the 15 Federal agencies that comprise ICCVAM and summarized ICCVAM's mission. He noted that ICCVAM, as an interagency committee, does not carry out research and development or validation studies. Instead, ICCVAM, in conjunction with NICEATM, carries out the critical scientific evaluation of proposed test methods with regard to their usefulness and limitations for regulatory testing and then makes formal recommendations to ICCVAM agencies.

Dr. Stokes provided a brief review of ICCVAM's history and summarized the ICCVAM Authorization Act of 2000,[2] detailing the purpose and duties of ICCVAM. He noted that one of ICCVAM's duties is to review and evaluate new, revised, and alternative test methods applicable to regulatory testing. He stated that all of the reports produced by NICEATM are available on the NICEATM-ICCVAM website or can be obtained upon request from NICEATM. He also mentioned that ICCVAM provides guidance on test method development, validation criteria, and processes, and helps to facilitate not only the acceptance of scientifically valid alternative methods, but also encourages international harmonization.

Dr. Stokes then described the ICCVAM test method evaluation process, which begins with a test method nomination or submission. NICEATM conducts a prescreen evaluation to summarize the extent to which the proposed submission or nomination addresses the ICCVAM prioritization criteria. A report of this evaluation is then provided to ICCVAM, which in turn develops recommendations regarding the priority for evaluation. ICCVAM then seeks input on their recommendations from the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) and the public. Given sufficient regulatory applicability, sufficient data, resources, and priority, a test method will move forward into a formal evaluation. A draft background review document (BRD), which provides a comprehensive review of all available data and information, is prepared by NICEATM, in conjunction with an ICCVAM working group designated for the relevant toxicity testing area (e.g., the IWG). In addition, ICCVAM considers all of the available information and makes draft test method recommendations on the proposed usefulness and limitations of the test methods, test method protocol, performance standards, and future studies. The BRD and the draft ICCVAM test method recommendations are made available to the Panel and the public for review and comment. The Panel peer reviews the BRD and evaluates the extent to which it supports the draft ICCVAM test method recommendations. A Panel report is published, which is then considered along with public and SACATM comments by ICCVAM in making final recommendations. These final recommendations are forwarded to the ICCVAM member agencies for their consideration and possible incorporation into relevant testing guidelines.

Dr. Stokes reviewed the ICCVAM criteria for adequate validation. He stated that validation is defined by ICCVAM as the process by which the reliability and relevance of a procedure are established for a specific purpose, and that adequate validation is a prerequisite for consideration of a test method by U.S. Federal regulatory agencies. Dr. Stokes listed the ICCVAM acceptance criteria for test method validation and acceptance. He concluded by summarizing the timeline of the review activities beginning with CPSC's nomination in January 2007 and ending with the present Panel meeting.

[1] http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf
[2] http://iccvam.niehs.nih.gov/docs/about_docs/PL106545.pdf

ICCVAM Charge to the Panel

Dr. Stokes reviewed the charge to the Panel, which was to: (1) review the draft BRDs, the draft Addendum to the traditional[3] LLNA, and the draft performance standards for completeness and identify any errors or omissions; (2) determine the extent to which each of the applicable criteria for validation and regulatory acceptance had been addressed for the proposed revised or modified versions of the LLNA; and (3) consider and provide comment on the extent to which the ICCVAM draft test method recommendations including the proposed use, standardized protocols, performance standards, and additional studies are supported by the information provided in the draft BRDs and draft Addendum.

Dr. Stokes thanked the IWG and ICCVAM for their contributions to this project, and acknowledged the contributions from the participating liaisons from ECVAM and JaCVAM (Japanese Center for the Validation of Alternative Methods). He also acknowledged the NICEATM staff for their support and assistance in organizing the Panel meeting and preparing the materials being reviewed.

Current Regulatory Testing Requirements and Hazard Classification Schemes for Allergic Contact Dermatitis and the Traditional LLNA Procedure

Dr. Joanna Matheson, Chair of the IWG, briefly reviewed the regulatory testing requirements of U.S. Federal agencies for skin-sensitization hazard identification and provided a brief description of the LLNA protocol.

Overview of the Agenda

Dr. Luster provided a brief synopsis of the agenda. He stated that there were six test methods and applications along with the draft LLNA performance standards for review and that the same agenda would be followed for each: (1) introductory summary of the draft ICCVAM recommendations from one of the NICEATM staff members; in addition, test method developers would provide a brief description of the methodology for each of the three nonradioactive tests, (2) presentation of the Evaluation Group draft comments by the Evaluation Group leader, (3) Panel discussion, (4) public comments, (5) recommendations and conclusions by the Panel.

Overview of the Draft LLNA Limit Dose Procedure[4] BRD and Draft ICCVAM Test Method Recommendations

Dr. David Allen, Integrated Laboratory Systems, Inc., the NICEATM support contractor, presented an overview of the draft ICCVAM BRD for the LLNA limit dose procedure. He mentioned that the draft ICCVAM BRD provided a comprehensive review of the available data and information regarding the usefulness and limitations of the LLNA limit dose procedure. The method was reviewed for its accuracy in correctly identifying sensitizers and non-sensitizers, when compared to the traditional LLNA.

NICEATM published a series of *Federal Register (*FR*)* notices, including an FR notice (72 FR 27815, May 17, 2007) requesting original data from the LLNA. This FR notice was also sent to over 100 potentially interested stakeholders for their input and comment. As a result, data on 255 substances tested in the LLNA were received. The resulting LLNA database consisted of 471 studies of 466 unique substances, 211 of which were included in the original ICCVAM 1999 evaluation. Dr. Allen briefly summarized the performance characteristics of the LLNA limit dose procedure test

[3] For the purposes of this document, the radioactive LLNA test method, which was first evaluated by ICCVAM in 1999, and subsequently recommended to U.S. Federal agencies as a valid substitute for currently accepted guinea pig test methods to assess the allergic contact dermatitis potential of many, but not all, types of substances, is referred to as the traditional LLNA.

[4] Also known as the reduced LLNA (rLLNA).

method, which is detailed in the draft ICCVAM BRD,[5] and briefly summarized the draft ICCVAM test method recommendations for the LLNA limit dose procedure.[6]

Panel Evaluation:

Dr. Michael Olson led the Panel discussion on the LLNA limit dose procedure and specifically thanked the members of his Evaluation Group (i.e., Drs. James McDougal, Raymond Pieters, Jonathan Richmond [not present], and Takahiko Yoshida) for their collegial review of the information presented in the draft ICCVAM LLNA Limit Dose Procedure BRD. Dr. Olson also thanked the NICEATM staff for their technical support during the BRD review process. He then presented the draft responses to ICCVAM's questions to the Panel for consideration by the entire Panel. The focus was on review of the BRD for errors and omissions, assessment of the validation status of the test method, and review of draft ICCVAM test method recommendations. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD and recommendations are reflected in the *Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products*, published in May 2008 (hereafter, the Panel report[7]).

During the Panel's evaluation, discussion arose regarding what might have resulted in the inverted-U-shaped dose response that was seen with the false-negative substances in the LLNA limit dose procedure. Dr. Olson responded that although it was difficult to understand what the cause might have been, he speculated that the top dose was either toxic at a systemic-effect level or that those substances were immunosuppressive at the highest dose level. He also stated that there did not seem to be any structural features of the substances that could be attributed for the false negative response in the LLNA limit dose procedure.

The Panel also discussed the use of concurrent versus intermittent positive controls in the LLNA limit dose procedure. Dr. Olson indicated that the Evaluation Group had discussed the possibility to allow intermittent positive controls for laboratories that exhibited repeatable and adequate performance with the LLNA but he indicated that it would be important to describe a set of performance criteria that would determine when this practice would be acceptable. Clearly, if the laboratory was not performing the assay routinely or if there were other reasons to suspect variability in response with any substance, the positive control would be necessary. Dr. Stokes indicated that this discussion was pertinent and indicated that the Panel's suggestions for what the performance criteria might be for intermittent positive control testing would be of interest to the IWG. Dr. Stokes also wanted to clarify that the OECD TG is consistent with the EPA TG and the ICCVAM-recommended test method protocol for the LLNA although the OECD TG allows additional latitude in how tests are run (i.e., four animals per dose group, use of pooled data, and the option to not run a positive concurrent positive).

Public Comments:

Dr. Amy Rispin, EPA

Dr. Rispin stated that the ICCVAM LLNA report (1999[8]) and standardized protocol (2001[9]) recommends the use of a concurrent positive control in addition to the concurrent negative control required for each study. Subsequently, the OECD (Organisation for Economic Co-operation and Development) Test Guideline (TG) 429 (Skin Sensitisation: Local Lymph Node Assay) was finalized (2002). She said that originally, OECD TG 429 was drafted without a concurrent positive control but that language was added to include the recommended use of a concurrent positive control until

[5] http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-LD/LLNAldBRD07Jan08FD.pdf
[6] http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-LD/IWGrecLLNA-LD07Jan08FD.pdf
[7] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[8] http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf
[9] http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/LLNAProt.pdf

laboratories demonstrate competence. Subsequent to that, EPA put forth its LLNA guideline for sensitization,[10] which states that concurrent positive and negative controls are to be included in each study. Dr. Rispin then added that U.S. Federal regulatory agencies, most notably the EPA and FDA, received LLNA data from studies in which the positive control did not achieve the appropriate limits of performance (i.e., the control values were not in the appropriate range) and therefore the studies were deemed unacceptable, underscoring the importance of a concurrent positive control for regulatory acceptance in the United States.

In response to Dr. Rispin's public comment, Drs. Ullrich and Theran asked how competence is determined and if laboratories have difficulties reaching a level of competence, respectively. Dr. Abby Jacobs responded by stating that the FDA has seen large data variations in laboratories that conduct the LLNA. It is often difficult to determine what the variations might be due to (e.g., new technicians, tail vein injection, lymph node removal) and these variations have been seen both in laboratories that are established and those that are not.

Dr. David Basketter, ECVAM Observer

Dr. Basketter said that the main point he wanted to address is that efforts should be made to harmonize the LLNA protocol with that described in OECD TG 429. He stated that although there is referral to the "ICCVAM protocol" throughout the BRDs under consideration, OECD TG 429 is more globally recognized for regulatory use of the LLNA and therefore should be the referenced protocol. Dr. Basketter further stated that if the LLNA limit dose procedure followed the ICCVAM protocol using five animals per group instead of following OECD TG 429, which allows using four animals per group, there would only be a savings of one animal for substances that were negative. He stated that the goal of ECVAM was actually to halve the number of animals by omitting the mid- and low-dose groups and that this would achieve significant animal savings since the likely prevalence of non-sensitizers is approximately two-thirds of chemicals tested and non-sensitizers would not require further testing even if dose response information for sensitizers was needed.

Dr. Basketter also mentioned that the retrospective evaluation of the LLNA being presented to the Panel analyzed whether the top dose could identify a substance as a sensitizer and how that compares to the traditional LLNA's performance. Since the traditional LLNA assay was determined to be positive or negative based on a stimulation index (SI) of three, it is problematic if the focus is on statistics when using the five-animal model as this would require also going back and re-evaluating all the preceding data using the statistical approach.

Dr. McDougal responded to Dr. Basketter's comment by stating that one wouldn't have to go back and retrospectively re-evaluate previous data but that new data generated could be analyzed statistically. This approach would include determining if the treatment group was statistically different from the vehicle control group and then determining the biological relevance. This might help to eliminate irritants.

Panel Conclusions and Recommendations:

Dr. Luster asked the Panel to review the conclusions and recommendations for the LLNA limit dose procedure they had discussed earlier and to make any revisions, if necessary. One particular question that was asked during the Panel's conclusions and recommendations was whether an OECD TG existed for the LLNA limit dose procedure. Dr. Stokes indicated that the OECD TG would need to be updated to allow for the provision of a limit dose procedure and that's why the Panel's conclusions and recommendations are even more relevant. Dr. Stokes indicated that ICCVAM has already submitted a proposal to update the OECD TG based on the outcome of these deliberations and recommendations from the IWG.

[10]http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Revised/870r-2600.pdf

The Panel agreed to use the term weight-of-evidence to refer to existing information that would aid the LLNA limit dose procedure in identifying a substance as a sensitizer or a non-sensitizer. The Panel also discussed the use of concurrent positive controls and recommended that a laboratory that is proficient at conducting the limit dose procedure can test a positive control at routine intervals rather than concurrently (although the Panel did not identify what constituted routine intervals). The Panel also discussed the use of individual versus pooled data and agreed with the ICCVAM-recommended protocol that individual animal data should always be collected. The Panel concluded that individual animal response data are necessary in order to allow for statistical analyses of any differences between treated and control data. In addition, having data from individual animals also allows for identification of technical problems and outlier animals within a dose group. Dr. Luster asked the Panel if they agreed with the changes and revisions made at this point and with the Panel conclusions and recommendations as presented and revised. The Panel unanimously agreed. The Panel's detailed recommendations and conclusions on the LLNA limit dose procedure are included in their final Panel report.[11]

Overview of the Draft Addendum for the Applicability Domain of the LLNA and Draft ICCVAM Test Method Recommendations

Dr. Eleni Salicru, Integrated Laboratory Systems, Inc. (the NICEATM support contractor), summarized the information provided in the draft ICCVAM Addendum to the ICCVAM LLNA report (1999). This Addendum provided an updated assessment of the validity of the LLNA for testing the sensitizing potential of mixtures, metals, and aqueous solutions. The database used for this evaluation contained traditional LLNA data submitted as part of the original LLNA evaluation (ICCVAM 1999), data extracted from peer-reviewed articles published after the original evaluation, and data submitted to NICEATM in response to the FR notice (72 FR 27815, May 17, 2007) requesting such data. Dr. Salicru then summarized the performance characteristics of the LLNA when used to test mixtures, metals, and aqueous solutions,[12] as well as the draft ICCVAM test method recommendations for each of the three categories of test substances.[13]

Panel Evaluation:

Dr. McDougal, on behalf of his Evaluation Group, presented for consideration by the entire Panel the draft responses to the questions asked of the Panel by ICCVAM. The Panel then discussed the completeness of the draft ICCVAM Addendum, identified any errors and omissions, and reviewed the draft ICCVAM test method recommendations with regard to the ability of the LLNA to be used to test the sensitizing potential of mixtures, metals, and aqueous solutions. The Panel discussion and their recommended revisions to each section of the draft ICCVAM Addendum are reflected in the Panel report, published in May 2008.[14] During the Panel's evaluation of the LLNA's applicability domain, the difficulty of testing metals in the LLNA was discussed and Dr. Woolhiser asked if testing metals was also problematic in the guinea pig. Dr. Api indicated that with the metals, most of the data has come from the clinical experience because animal studies are not predicting accurately what is happening in the clinic. Dr. Maibach indicated that metals have been tested in the guinea pig and that they are sensitized easily. Dr. Maibach further commented that metals in man need to be patch-tested for clinical relevance at a level close to the irritant dose and that a thoughtful series of algorithms is necessary to determine this. He also pointed out that patch test results to some metals (e.g., nickel, palladium) may indicate that a cell mediated reaction is occurring (i.e., contact allergy) but it needs to be sorted out if this cell mediated reaction actually results in a disease (i.e., allergic contact dermatitis) and this is where the LLNA could prove useful.

[11] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[12] http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-app/LLNAappADD19Jan08FD.pdf
[13] http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-app/LLNAappRecs19Jan08FD.pdf
[14] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

With regard to mixtures, Dr Api commented that based on her experience, when the mixture tested in the LLNA contains a predominant material (loosely defined that as greater than 70 percent) then the LLNA for the mixture mirrors what occurs for that one material. When evidence indicates that the substance is a true mixture, some times the LLNA does what is expected and other times the results are unexpected. In those cases, a weight-of-evidence approach (e.g., structure-activity relationships, clinical evidence) is employed.

Public Comments:

Dr. Charles Hastings, BASF Corporation

Dr. Hastings, representing CropLife America (an industry association of companies in the crop protection business), provided an overview of current activities in industry related to the use of the LLNA to detect dermal sensitizers and the global issues that are of importance. Dr. Hastings mentioned that CropLife America's primary concern is the testing of pesticide mixtures and formulations. He stated that they support the use of the LLNA for testing the dermal sensitization of mixtures and formulations as well as single ingredients.

Dr. Hastings mentioned that in the United States, EPA OPPTS (Office of Prevention, Pesticides and Toxic Substances) Guideline 870.2600[15] allows for the use of the LLNA as the preferred alternative to the standard guinea pig test. Based on this recommendation, member companies of CropLife America conducted a large number of LLNA studies for both active ingredients and formulations in the European Union (E.U.) and were at the point of submitting data in the United States, as well. Then, in early 2007, they were informed that EPA had concerns about the validity of using the LLNA to test mixtures and formulations, and were advised to discontinue using this test method for that purpose until it had been adequately validated. Dr. Hastings stated that, in contrast to the EPA, E.U. regulators consider the LLNA acceptable for testing pesticide formulations and actually prefer it to a guinea pig test.

Dr. Pieters asked if the E.U. has conducted any evaluations of the validity of the LLNA for testing mixtures and formulations. Dr. Hastings replied that he was not certain if they had performed an extensive evaluation or not but that the E.U. considered the LLNA a validated method and therefore likely considered it appropriate to test not only the active ingredient but also the formulation or mixture.

Dr. Hastings mentioned that one concern in terms of using the LLNA for testing mixtures or formulations, particularly in the E.U., is the testing of aqueous substances. Many of the industry formulations are aqueous-based and may be incompatible with traditional LLNA vehicles. The European Crop Protection Association sponsored a study that evaluated the use of an aqueous vehicle known as Pluronic L92, which helps adhere the test material to the mouse ear. In the study, they tested three aqueous pesticide formulations that contained known sensitizers, using Pluronic L92 as the vehicle. As expected, the test results demonstrated sensitizing activity. Regarding global considerations, Dr. Hastings mentioned that if the LLNA is not accepted for mixture/formulation testing in the United States, industry will have no choice but to conduct both the LLNA, with 18 to 24 animals, and a guinea pig test, with 20 to 30 animals, for each formulation they may develop for global distribution. This scenario counters the ICCVAM goal of "reducing, refining, and replacing" animal use in regulatory safety testing.

Dr. Hastings ended with the following conclusions:

- CropLife America believes the LLNA test can be used for pesticide formulations.

[15]http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Revised/870r-2600.pdf

- CropLife America supports the efforts of EPA and ICCVAM to confirm the validity of the LLNA for testing mixtures/formulations and encourages a quick evaluation.
- CropLife America is willing to help, as needed.
- If and, when, it is determined that the LLNA is acceptable, CropLife America requests that EPA notify them so they can then begin conducting the LLNA again for the United States.

Dr. Api asked if CropLife America has data comparing pesticides that have been evaluated in the LLNA and in guinea pigs and/or humans. Dr. Hastings replied that they do and that generally there is not much discrepancy with guinea pig test results. Occasionally they might see a false positive compared to a guinea pig test, but he did not recall ever seeing a false negative. In most cases, they would feel comfortable accepting an occasional false positive because human health is still protected.

Dr. David Basketter, ECVAM Observer

Dr. Basketter stated that he had personal reservations about testing complex mixtures and formulations in assays that were designed for testing substances (e.g., the LLNA) since no single test has ever been validated for testing mixtures. On another point, he stated that most of the metals of importance have been tested in both the guinea pig and the LLNA and the "right" answers have been generated. Thus, it does not seem worthwhile to produce new tests with revised protocols for hazard and potency categorization for testing metals.

Panel Conclusions and Recommendations:

Dr. Luster asked the Panel if they agreed with the comments and recommendations that were made earlier during the Panel discussion. The Panel agreed with the draft ICCVAM recommendation for continued collection of information from traditional LLNA evaluations of mixtures, metals, and aqueous solutions with comparative data for guinea pig (i.e., guinea pig maximization test [GPMT] or Buehler test [BT]) and human (i.e., human maximization test [HMT] or human repeat insult patch test [HRIPT]) tests. However, the Panel suggested that, given resource limitations, it would be important to organize the recommendations based on relative priority. Dr. Luster asked the Panel if they agreed with this suggestion about prioritization of activities; all members of the Panel agreed with one abstention. Dr. Howard Maibach abstained from voting stating that he hoped this public meeting and the subsequent Panel report would emphasize to industry the need for them to submit more data on mixtures, metals, and aqueous substances in order to provide a clearer evidence of the validity of the LLNA in testing these types of substances. The Panel's detailed recommendations and conclusions on the applicability domain of the LLNA are included in their final Panel report.[16]

Method Description and Overview of the LLNA: Daicel Adenosine Triphosphate (LLNA: DA) Test Method

Dr. Kenji Idehara, Daicel Chemical Industries, Ltd. (private limited company), summarized the technical aspects of the LLNA: DA test method. He described the LLNA: DA as a non-radioisotopic version of the LLNA method in which lymph node adenosine triphosphate (ATP) content is used as a measure of cell proliferation instead of radiolabeled thymidine incorporation. Dr. Idehara indicated that the LLNA: DA was developed six years ago at Daicel Chemical Industries, Ltd., and that they use the test method regularly for in-house assessments of the skin-sensitization potential of chemical materials, intermediates, or products. He summarized the protocol differences between the LLNA: DA and the traditional LLNA. In the LLNA: DA, the application site is treated with 1% sodium lauryl sulfate (SLS) one hour before each test substance (or vehicle control) application, and the test substance is applied to the test site on day 7 as well as on days 1, 2, and 3. The auricular lymph nodes are excised from individual animals on day 8 rather than on day 6 and the amount of ATP in the

[16] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

lymph nodes is measured with a luciferin-luciferase assay. Dr. Idehara mentioned that these modifications (i.e., 1% SLS pretreatment and additional application on day 7) enhance lymph node cell proliferation in order to achieve an SI = 3 in the LLNA: DA, which allows for a more direct comparison to the traditional LLNA.

Dr. Idehara mentioned that after excision, ATP content gradually decreased with time. Therefore, the overall assay time for measuring ATP content needs to be similar (i.e., within approximately 30 minutes) among all test animals. He noted that this was an important point for this method and recommended that the LLNA: DA be conducted by at least two persons. Dr. Idehara mentioned that ATP content assays are conducted using commercially available kits, and his laboratory has experience with two different commercial sources in Japan, Kikkoman and Lonzar.

Overview of the Draft LLNA: DA BRD and Draft ICCVAM Test Method Recommendations

Dr. Allen then presented an overview of the draft ICCVAM BRD for the LLNA: DA test method. He mentioned that the draft ICCVAM BRD provided a comprehensive review of the available data and information regarding the usefulness and limitations of the LLNA: DA to distinguish between sensitizers and non-sensitizers, compared to the traditional LLNA. The objective of the BRD was to describe the current validation status of the LLNA: DA test method, including its relevance and reliability, scope of substances tested, and the availability of a standardized protocol.

Dr. Allen mentioned that the data analyzed in the BRD included data provided by Daicel Chemical Industries, Ltd., on 31 substances tested at their laboratories. In addition, data for 14 different coded substances were generated from a two-phased interlaboratory validation study that included 17 total labs. Taken together, the total database represented in the LLNA: DA BRD included 33 different substances. Dr. Allen briefly summarized the performance characteristics of the LLNA: DA test method, which is detailed in the draft ICCVAM BRD.[17] Dr. Allen concluded by briefly summarizing the draft ICCVAM test method recommendations for the LLNA: DA test method.[18]

Panel Evaluation:

Dr. Michael Woolhiser thanked the Panel members of his Evaluation Group (i.e., Drs. Nathalie Alépeé, Thomas Gebel, Sidney Green [not present], and Jean Regal) for their tireless efforts in reviewing their Evaluation Group's assigned documents. He also thanked the NICEATM staff for their technical support during the review process. Dr. Woolhiser then presented the draft responses to ICCVAM's questions about this test method for consideration by the entire Panel. This included their review of the draft BRD for errors and omissions, their overall assessment of the validation status of the test method, and their comments on the draft ICCVAM test method recommendations. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD are reflected in the Panel report, published in May 2008.[19]

Adjournment—

The meeting was adjourned for the day at 5:03 p.m., to reconvene at 8:30 a.m., Wednesday, March 5, 2008.

[17] http://iccvam.niehs.nih.gov/methods/immunotox/llna-DA/LLNA-DAbrd07Jan08FD.pdf
[18] http://iccvam.niehs.nih.gov/methods/immunotox/llna-DA/LLNA-DARecs07Jan08FD.pdf
[19] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

WEDNESDAY, MARCH 5, 2008

Reconvening of the Panel Meeting

Dr. Luster reconvened the Panel Meeting at 8:30 a.m. He introduced himself and then asked that all Panel members, followed by all others in attendance, introduce themselves as well.

Overview of the Draft LLNA: DA BRD and Draft ICCVAM Test Method Recommendations

Panel Evaluation:

Dr. Woolhiser continued his presentation from the previous day of the draft responses to ICCVAM's questions to the Panel, for consideration by the entire Panel. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD are reflected in the Panel report, published in May 2008.[20] Dr. Woolhiser indicated that the Evaluation Group had two main concerns with the LLNA: DA test method. The first concern related to pretreatment with 1% SLS and understanding how this impacted the biology of the response. Second, the time course of the study was different than the traditional LLNA because it extended the study by one day and included an additional challenge. This brought forth a question about the immunology of the response as it relates to the potential for elicitation and whether or not that is a significant change from the traditional LLNA, which is purely an induction model.

Public Comments:

Dr. George DeGeorge, MB Research Laboratories
In response to a question raised during the Panel discussion, Dr. DeGeorge commented that using lymph node weight as the readout to differentiate between sensitizers and non-sensitizers in the LLNA is problematic because although there are more lymph node cells packed into a node, each cell has less cytoplasm. The lymph nodes swell to a point, and then excrete water and become smaller lymphocytes that are countable. He cited examples from his laboratory with several different sensitizers, which demonstrate that lymphocytes in the node are smaller when a large SI (e.g., SI = 25) is obtained relative to when a smaller SI (e.g., SI = 3) is obtained.

Dr. DeGeorge also commented that he agreed with a point made during the Panel discussion that the LLNA: DA method and the LLNA: Bromodeoxyuridine Detected by ELISA (LLNA: BrdU-ELISA) method should be considered separately, because they are so dissimilar.

In his final comment, Dr. DeGeorge stated that in the traditional LLNA, in the LLNA: Bromodeoxyuridine Detected by Flow Cytometry (LLNA: BrdU-FC), and probably also in the LLNA: DA, strong sensitizing substances do not need to be administered three times. For instance, if one administers a single, moderately high dose of dinitrochlorobenzene (DNCB) (i.e., one that would induce an SI of 20 to 40) and then measures lymph node cell proliferation on day 1, 2, 3, or 4, an increase in the number of cells in the node and the number of cells that are positive for BrdU would likely be observed. Thus, administrations of additional applications have the potential to cause cumulative irritation. Dr. DeGeorge stated that the LLNA: DA method, which extends the assay to eight days instead of six days, should evaluate what happens to lymph node cell number at earlier sample times. In addition, if the animals receive just one application using a high dose, with or without the SLS, is there an increase in the SI? If so, that would lead to the possibility that the extra applications are not necessary and might lead to cumulative irritation.

Dr. David Basketter, ECVAM Observer
Dr. Basketter made a statement that from a clinical perspective, substances are typically described as

[20] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

significant sensitizers or not significant sensitizers, and within that latter group some of the substances may indeed be non-sensitizing. Thus, just because a substance has been shown in an isolated case report to be a human sensitizer does not mean that there is sufficient evidence to consider it as positive for comparison with outcomes of predictive assays. It has to be of sufficient importance (i.e., potency) to trigger a positive classification. Dr. Basketter mentioned SLS, methyl salicylate, and isopropanol, as substances which will always be positive in some human cases although they shouldn't be positive in a predictive assay.

Dr. Basketter also commented that caution should be given to making sensitization assumptions based on chemical class references. As an example, eugenol and isoeugenol are structurally similar and have similar physical properties, but they act by different chemical reaction mechanisms and could fit into distinctly different chemical classes.

Dr. Basketter's last comment acknowledged that much work has been done in terms of validating the traditional LLNA. If one makes minor changes to the LLNA in terms of a different readout for proliferation, then they benefit from all the experience generated in validating the traditional LLNA and less effort is needed to prove that the minor modification is valid. In contrast, if more significant modifications are made, one cannot rely on that same experience. Dr. Basketter cautioned that more importance should be placed on distinguishing whether something has changed substantially enough such that you can no longer rely on the traditional LLNA as a reference.

Dr. Masahiro Takeyoshi, Chemicals Evaluation and Research Institute

Dr. Takeyoshi made a short presentation about differences in LLNA sensitization responsiveness among different strains of mice. He mentioned that this was an important issue when evaluating the modified LLNA methods being developed in Japan. He showed differences in responsiveness among three different mouse strains commonly used in Japan (i.e., BALB/cAnN, CBA/JN, and CD-1) tested with parabenzoquinone in his group's non-radioactive LLNA (i.e., LLNA: BrdU-ELISA). The data indicated that the CBA/JN mouse strain exhibited a higher responsiveness, as indicated by an increased SI, to parabenzoquinone than the other two mouse strains tested. Based on these results, CBA/JN mice were chosen for testing substances in the LLNA: BrdU-ELISA test method.
Dr. Takeyoshi also indicated that based on evaluating different SI cutoffs in the LLNA: BrdU-ELISA, 2-mercaptobenzothiazole, 3-(4-isopropylphenyl)isobutyraldehyde, and hydroxycitronellal had low responsiveness (i.e., SI values). He noted that 2-mercaptobenzothiazole is an OECD TG 429 recommended positive control for the LLNA; however, repeat tests could not detect this substance as positive when using an SI value of 1.7 or more. Dr. Takeyoshi suggested that a substance-specific lower response might exist in the test system. Dr. Takeyoshi also summarized LLNA data by Dr. Ullmann and coworkers with the contract lab RCC, Ltd. in which they investigated the responsiveness of six different mouse strains (CBA/CaOlaHsd, CBA/Ca (CruBR), CBA/Jlbm (SPF), CBA/JNCrj, BALB/c and NMRI) to 25% 2-mercaptobenzothiazole. The data indicated that CBA/JNCrj mice showed markedly lower responsiveness compared to the other strains tested. These studies indicate that strain related differences would not be negligible with regard to measuring different endpoints of cellular proliferation in the LLNA because depending on the chemicals tested, responsiveness might be potentially impacted. For instance, some of the discordance seen in the LLNA: DA test method (e.g., 2-mercaptobenzothiazole) could be a strain specific effect.

Panel Conclusions and Recommendations:

Dr. Luster asked the Panel to review their conclusions and recommendations and discuss any revisions, if necessary. The Panel viewed the difference in treatment schedule between the LLNA: DA and the traditional LLNA to potentially be significant if the treatment schedule for the LLNA: DA corresponds to entering the elicitation phase of skin sensitization. The Panel was concerned that the 1% SLS pretreatment step in the LLNA: DA might modify the inherent sensitivity of the LLNA. They recommended that the test method developer (Daicel Chemical Industries, Ltd.) justify the use of 1% SLS or consider an alternative decision criterion (i.e., an SI threshold other than three) such

that the 1% SLS pretreatment is no longer necessary. Dr. Luster asked the Panel if they agreed with the recommendations and conclusions that the Panel made along with the revisions; unanimously, the Panel agreed. The Panel's detailed recommendations and conclusions on the LLNA: DA test method are included in their final Panel report.[21]

Method Description and Overview of the LLNA: BrdU-FC Test Method

Dr. George DeGeorge, MB Research Laboratories, presented an overview of the LLNA: BrdU-FC test method. He stated that mice are dosed topically on the ears once daily for three consecutive days (i.e., days 1, 2, and 3), just like the traditional LLNA protocol. On day 6, the mice receive an intraperitoneal injection with bromodeoxyuridine (BrdU), and five hours later, the auricular lymph nodes are removed. The lymph nodes from individual animals are processed and, using flow cytometry, the number of BrdU-positive cells are counted from treated animals and compared to control animals as a measure of lymph node cell proliferation.

Dr. DeGeorge described in detail how the cells are processed and gated for flow cytometric analysis. He mentioned that the cells are also permeabilized and treated with propidium iodide which allows gates to be drawn around the G_0, G_1, S, and G_2M phases of the cell cycle. Dr. DeGeorge projected specific examples of flow cytometry plots and histograms for DNCB, hexyl cinnamic aldehyde (HCA), and positive and negative control data.

Dr. DeGeorge also described the tiered protocol for the assessment of sensitization potential using the LLNA: BrdU-FC and how ear swelling measurements and additional immunophenotypic endpoints (i.e., the enhanced LLNA: BrdU-FC) aid in distinguishing skin irritants from an irritating sensitizer.

Overview of the Draft LLNA: BrdU-FC BRD and Draft ICCVAM Test Method Recommendations

Dr. Judy Strickland, Integrated Laboratory Systems, Inc. (the NICEATM support contractor), presented an overview of the draft ICCVAM BRD for the LLNA: BrdU-FC test method. She stated that the draft ICCVAM BRD provided a comprehensive review of the available data and information regarding the usefulness and limitations of the LLNA: BrdU-FC test method. Specifically, the test method was reviewed for its ability to distinguish between sensitizers and non-sensitizers compared with the traditional LLNA. The objective of the BRD was to describe the current validation status of the LLNA: BrdU-FC test method, including its relevance and reliability, scope of substances tested, and the availability of a standardized protocol.

Dr. Strickland indicated that MB Research Laboratories submitted data to NICEATM for the 48 substances analyzed in the BRD in response to an FR notice (72 FR 27815, May 17, 2007) that requested such data. Dr. Strickland briefly summarized the performance characteristics of the LLNA: BrdU-FC test method, which is detailed in the draft ICCVAM BRD,[22] and the draft ICCVAM test method recommendations for the LLNA: BrdU-FC test method.[23]

Panel Evaluation:

Dr. Raymond Pieters, on behalf of his Evaluation Group, presented the Evaluation Group's review of the draft BRD and the draft test method recommendations for the LLNA: BrdU-FC test method. Specifically, he presented the draft responses to ICCVAM's questions to the Panel for consideration by the entire Panel. This included their review of the draft BRD for errors and omissions, their overall assessment of the validation status of this test method, and their comments on the draft ICCVAM test

[21] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[22] http://iccvam.niehs.nih.gov/methods/immunotox/fcLLNA/FC-LLNAbrd07Jan08FD.pdf
[23] http://iccvam.niehs.nih.gov/methods/immunotox/fcLLNA/FCLLNARecs07Jan08FD.pdf

method recommendations. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD are reflected in the Panel report, published in May 2008.[24] The applicability of the draft ICCVAM-recommended LLNA performance standards to the LLNA: BrdU-FC test method was discussed, particularly with regard to the number of substances tested in the LLNA: BrdU-FC method and whether more data would be necessary for review before the validation status of the assay could be determined. Dr. Stokes reminded the Panel that the proposed LLNA performance standards didn't exist when the studies for the LLNA: BrdU-FC test method were performed. The questions should be whether the adequacy of the substances that have been tested is sufficient or if more studies need to be done to cover any gaps that might exist (e.g., range of potencies or activity, chemical classes).

Public Comments

Dr. David Basketter, ECVAM Observer

Dr. Basketter commented on the statement that Dr. DeGeorge made during his overview of the LLNA: BrdU-FC test method that HCA is irritating. He said that he is not convinced it is a significant irritant. Based on previous data, they had to use 50% HCA in a 48 hour occlusive application in the guinea pig in order to produce a mildly irritating response. Dr. Api added to Dr. Basketter's comment by stating that RIFM has also not found HCA to be an irritant when tested up to 20% in humans.

Dr. Basketter also commented that in the draft BRD for the LLNA: BrdU-FC, resorcinol was noted to be negative in the traditional LLNA and this is not correct. Dr. Basketter's group published results in 2007 in the journal Contact Dermatitis that resorcinol is clearly positive in the traditional LLNA when tested at higher concentrations and therefore this should be corrected for the record.

Dr. George DeGeorge, MB Research Laboratories

Dr. DeGeorge wanted to clarify that the LLNA: BrdU-FC test method was compared to the traditional LLNA to determine if the LLNA: BrdU-FC was more predictive of skin-sensitization potential. He stated that in some cases it was better while in others it wasn't, but overall, using human data as the gold standard reference, the LLNA: BrdU-FC exceeded the traditional LLNA predictivity values and accuracy. He also noted that the additional endpoints included in the LLNA: BrdU-FC allow for them to distinguish irritating substances that typically are considered false positives in the LLNA.

Dr. DeGeorge also noted that since the LLNA: BrdU-FC is so similar to the traditional LLNA the issue of refinement and reduction in animal use is not immediately apparent but if the assay is done in as few as four mice per group with a periodic positive control (e.g., every six months) this represents a significant decrease in animal numbers compared to guinea pig tests. Furthermore, there is a refinement since mice are phylogenetically lower than guinea pigs, and undergo less pain and distress during the assay than guinea pigs undergo.

With regard to the discussion of coefficients of variation (CVs) and the 0.5x to 2.0x EC3 (i.e., the estimated concentration needed to produce a stimulation index of three) range, Dr. DeGeorge suggested that a larger range might be more reasonable because the current range is likely too restrictive.

Dr. George also noted that ICCVAM requires interlaboratory validation if a test method is to be transferred to other laboratories. With regard to the LLNA: BrdU-FC, it is a "me-too" assay and only has "minor" changes from the traditional LLNA and is currently only used in one laboratory. Therefore, the current dataset should suffice for determining the validity of the LLNA: BrdU-FC. In response to Dr. DeGeorge's comment, Dr. Stokes stated that if a method is only proposed to be used by one laboratory, having only intralaboratory data certainly would suffice but if it was proposed for broader use (e.g., adopted or endorsed by regulatory authorities), then other laboratories would have to demonstrate

[24] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

interlaboratory reproducibility. Dr. Luster asked if there was any mechanism available so that a company or small laboratory could apply for funding to help support an interlaboratory validation. Dr. Stokes indicated that they could nominate the test method for additional validation studies to ICCVAM. It would go through a nomination review process and a prioritization would be given to that. The nomination would then be considered by the member agencies as to whether funding would be provided.

Panel Conclusions and Recommendations:

Dr. Luster asked the Panel to review their conclusions and recommendations and discuss any revisions, if necessary. The Panel suggested that the utility of ear swelling or other methods to detect inflammation appeared warranted for inclusion in every variation of the LLNA (including the traditional LLNA), but should be further investigated before routine inclusion in the protocol is recommended. The Panel further agreed that the draft ICCVAM test method recommendations for future studies highlighted the unanswered questions raised by the available data set. Specifically, conducting interlaboratory studies as a part of the validation process is important.

The Panel considered the immunological markers suggested for the LLNA: BrdU-FC to be appropriate, but noted that other immunological markers for discrimination of irritant versus sensitization phenomena were also available. In general, for any future work, efforts should be made to decrease the variability and to thereby increase the power of the test in order to ensure that more animals were not needed relative to the traditional LLNA or other modified LLNA protocols.

Dr. Luster asked the Panel to indicate if they agreed with the recommendations and conclusions that the Panel made along with the revisions; the Panel unanimously agreed. The Panel's detailed recommendations and conclusions on the LLNA: BrdU-FC test method are included in their final Panel report.[25]

Method Description and Overview of the LLNA: BrdU-ELISA Test Method

Dr. Masahiro Takeyoshi, Chemicals Evaluation and Research Institute, presented an overview of the LLNA: BrdU-ELISA test method. He stated that the LLNA: BrdU-ELISA test method is very similar to the traditional LLNA test method. Unique to the LLNA: BrdU-ELISA test method, after test substance applications on days 1, 2, and 3, BrdU is injected interperitoneally on day 5. Approximately 24 hours after the BrdU injection, lymph nodes are collected, and detection of the amount of BrdU incorporated into the DNA of lymph node cells is conducted with an ELISA.

In the development process of this method, experiments were conducted to detect the most efficient injection schedule of BrdU. Based on the various injection schedules tested, a single injection protocol on day four was identified as the optimal injection schedule for BrdU administration.

Dr. Takeyoshi then showed a video of laboratory personnel preparing the lymph node cells for BrdU detection by ELISA. He went on to describe data for the LLNA: BrdU-ELISA compared to the traditional LLNA and how performance could be improved using alternative decision criteria (i.e., an SI other than three as the threshold for a positive response).

Overview of the Draft LLNA: BrdU-ELISA BRD and Draft ICCVAM Test Method Recommendations

Dr. Salicru presented an overview of the draft ICCVAM BRD for the LLNA: BrdU-ELISA test method. She noted that the draft ICCVAM BRD provided a comprehensive review of the available data and information regarding the usefulness and limitations of the LLNA: BrdU-ELISA test method. Specifically, the test method was reviewed for its ability to distinguish between sensitizers

[25] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

and non-sensitizers compared with the traditional LLNA and guinea pig test methods. The objective of the BRD was to describe the current validation status of the LLNA: BrdU-ELISA test method, including its relevance and reliability, scope of substances tested, and the availability of a standardized protocol.

Dr. Salicru stated that data from a total of 29 substances were considered in the accuracy analysis for the LLNA: BrdU-ELISA, and they were all tested in one laboratory. Dr. Salicru briefly summarized the performance characteristics of the LLNA: BrdU-ELISA test method, which are detailed in the draft ICCVAM BRD,[26] and the draft ICCVAM test method recommendations for the LLNA: BrdU-ELISA test method.[27]

Panel Evaluation:

Ms. Kim Headrick presented her Evaluation Group's (Drs. Anne Marie Api, Howard Maibach, Peter Theran, and Stephen Ullrich) review of the draft BRD and draft ICCVAM test method recommendations for the LLNA: BrdU-ELISA test method. Specifically, she presented the draft responses to ICCVAM's questions to the Panel for consideration by the entire Panel. This included their review of the draft BRD for errors and omissions, their overall assessment of the validation status of the test method, and their comments on the draft ICCVAM test method recommendations. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD are reflected in the Panel report, published in May 2008.[28]

Public Comments:

Dr. David Basketter, ECVAM Observer
Dr. Basketter noted that when the traditional LLNA was first suggested as an alternative to the guinea pig tests, it went through a comprehensive validation process, and one of the concerns was that it should perform reliably and distinctly better than the guinea pig assays. He emphasized that this point should be kept in mind when thinking about the modified LLNA protocols with alternative endpoints that are currently being reviewed. He stated that the current rigor of examination for the modified LLNA protocols being reviewed for validation is higher than that for the traditional LLNA. He speculated that in the not-too-distant future, *in vitro* alternatives are likely to be going through a similar review process and it is going to become ever more difficult to put these alternatives in place, not because there is ill-will against the selections but because of the high standard of being good scientists. Thus, it is important that pragmatic decisions are made using the tools that are available.

Dr. George DeGeorge, MB Research Laboratories
Dr. DeGeorge commented that he agreed with Dr. Basketter's statements. He said that based on his experience in this peer review process, it is unlikely that he would bring any of the three *in vitro* test methods that MB Research Laboratories is developing for consideration by ICCVAM, given the many high hurdles that have to be negotiated.

In response to the comments by Drs. Basketter and DeGeorge, Dr. McDougal commented that it does not seem unreasonable to raise the bar for what is expected of new or modified tests. Dr. Luster added that understandably, the focus on animal refinement and reduction is paramount, but that as scientists we have to ensure that the bar is maintained sufficiently high so that as the years go by scientific quality is not compromised.

Panel Conclusions and Recommendations:

Dr. Luster asked the Panel to review their conclusions and recommendations and discuss any revisions, if necessary. The Panel concluded that the available data and test method performance for

[26] http://iccvam.niehs.nih.gov/methods/immunotox/llna-ELISA/BrdUELISAbrd07Jan08.pdf
[27] http://iccvam.niehs.nih.gov/methods/immunotox/llna-ELISA/BrdUELISARecs07Jan08FD.pdf
[28] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

the LLNA: BrdU-ELISA support the draft ICCVAM test method recommendations that it may be useful for identifying substances as potential skin sensitizers and non-sensitizers, but that more information and existing data must be made available before the LLNA: BrdU-ELISA can be recommended for use. The Panel also stated that a detailed protocol was needed, in addition to sufficient quantitative data for broader analysis on a larger set of balanced reference substances that take into account physicochemical properties and sensitization potency, as well as an appropriate evaluation of interlaboratory reproducibility.

The Panel's main concern with this test method was that the accuracy of the LLNA: BrdU-ELISA at SI ≥ 3 was inadequate and not equivalent to the traditional LLNA. Furthermore, although using a decision criterion of SI ≥ 1.3 improved the test's performance in identifying sensitizers from non-sensitizers, it did not resolve concerns about the test method, particularly considering that power calculations suggest a much larger number of animals per group would be required to identify a positive response. Thus, the Panel also concluded that it might be more appropriate to use a statistically based decision criterion rather than a stimulation index to classify substances as sensitizers, and that this should be further investigated. Dr. Luster asked the Panel to indicate if they agreed with the recommendations and conclusions that the Panel made along with the revisions; unanimously, the Panel agreed. The Panel's detailed recommendations and conclusions on the LLNA: BrdU-ELISA test method are included in their final Panel report.[29]

Overview of the Draft ICCVAM Performance Standards for the LLNA

Dr. Allen presented an overview of the draft ICCVAM Performance Standards for the LLNA. He briefly summarized the overall purpose of performance standards (i.e., to provide a basis for evaluating the performance of a proposed test method that is mechanistically and functionally similar to the validated test method) and the three elements encompassed within such performance standards (i.e., essential test method components, a minimum list of reference substances, and accuracy/reliability values). He noted that the proposed applicability of these draft ICCVAM LLNA performance standards is for the evaluation of LLNA protocols that deviate from the ICCVAM-recommended LLNA protocol only with respect to the method for assessing lymphocyte proliferation (e.g., using non-radioactive instead of radioactive reagents). Dr. Allen then provided an overview of the essential test method components, the minimum list of reference substances, and the accuracy/reliability values as detailed in the draft ICCVAM LLNA Performance Standards.[30]

Panel Evaluation:

Dr. Woolhiser, on behalf of his Evaluation Group, presented the Evaluation Group's responses to the ICCVAM questions asked about the draft ICCVAM LLNA Performance Standards for the entire Panel to consider. The overall question for the Panel was whether these performance standards were considered adequate for assessing the accuracy and reliability of test method protocols that were based on similar scientific principles and that measured the same biological effect as the traditional LLNA. The Panel discussion and their recommended revisions to the draft ICCVAM LLNA Performance Standards are reflected in the Panel report published in May 2008.[31]

Adjournment—

The meeting was adjourned at 5:42 p.m., to reconvene at 8:30 a.m., Thursday, March 6, 2008.

[29] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[30] http://iccvam.niehs.nih.gov/methods/immunotox/PerfStds/LLNAPerfStd07Jan08FD.pdf
[31] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

THURSDAY, MARCH 6, 2008

Reconvening of the Panel Meeting

Dr. Luster reconvened the Panel Meeting at 8:30 a.m. He introduced himself and then asked that all Panel members and all others in attendance introduce themselves as well.

Overview of the Draft ICCVAM LLNA Performance Standards

Panel Evaluation:

Dr. Woolhiser reviewed some of the important points highlighted during the previous day's discussion on this topic, and then continued to summarize the remaining comments of his Evaluation Group on the questions asked by ICCVAM on the draft ICCVAM LLNA Performance Standards for consideration by the entire Panel. As mentioned above, the Panel discussion and their recommended revisions to the draft ICCVAM LLNA Performance Standards are reflected in the Panel report published in May 2008.[32]

Dr. Woolhiser noted that there were general comments on the topic order for the Panel's review. He asked if Dr. Stokes would comment on the rationale for the topic order. Dr. Stokes indicated that as the IWG deliberated the order of topics for this review, consideration was given to the fact that the three non-radioactive methods had undergone validation studies prior to the creation of LLNA performance standards. Thus, the non-radioactive test methods were reviewed before the performance standards, so as to not bias the Panel's assessment of each test method's performance. The performance standards could then be considered for their application to future test methods.

Public Comments:

Dr. Amy Rispin, EPA

Dr. Rispin stated that her intent was to provide some additional regulatory perspective on some of the points that have been discussed. When Federal agencies evaluate the validation status of a test method under ICCVAM, they conduct a comprehensive analysis of overall performance (i.e., accuracy and reliability) in the context of making regulatory decisions with data from the test method. Thus, in a regulatory situation, equal or greater accuracy compared to the reference test method is the expectation. If the number of animals can be decreased only at the expense of accuracy, the acceptability of such a test method for the particular regulatory purpose would need to be carefully considered. Certain methods, instead of being complete replacements, might have to be relegated to the role of screens, where positives would be accepted, but negatives would require further testing - a less than ideal situation.

Dr. Rispin commented that performance standards are the regulating agencies' basis for the acceptability of variations of accepted test methods. If an agency receives data from a modified LLNA method that has not been reviewed and validated in the ICCVAM process, there is unlikely to be a comprehensive peer review of it within the agency, given resource limitations. Therefore, the question of major versus minor departures from the functional criteria is important to ICCVAM and its member agencies. One cannot anticipate that there will be anything other than these performance standards to adequately evaluate the usefulness and limitations of a new method.

Dr. David Basketter, ECVAM Observer

Dr. Basketter first commented on a point that Dr. Thomas Gebel alluded to during the Panel's discussion of the draft ICCVAM LLNA Performance Standards, which was that if a new laboratory performed the traditional LLNA to assess 18 or 22 chemicals, they probably wouldn't get a complete match. Dr. Basketter disagreed with Dr. Gebel's statement and viewed that a competent laboratory performing the LLNA would get it 100% correct.

[32] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

Dr. Basketter then provided some comments that he stated were "from the ECVAM perspective." He stated that the ECVAM performance standards tried to address adhering to a standard protocol and that any change to the protocol other than the method for evaluating lymph node proliferation (e.g., strain, species, number of applications, time) was considered not to be minor, and therefore such a protocol would not be applied to these performance standards. By restricting the performance standards to minor changes, ECVAM was trying to minimize the number of chemicals required to evaluate sensitivity. Furthermore, the EC3 value could be used to see if the test method could classify substances in the appropriate range of sensitization potency.

ECVAM initially chose their reference substances in order to determine whether a modified method (differing only in the method for measuring cell proliferation) would give the same answer as the traditional LLNA. Thus, there was no intent to compare to the guinea pig or human data.

Dr. Basketter speculated that it is doubtful that data from multiple LLNA studies on the same substance are available and therefore it is unlikely that much larger sample sizes from which to calculate mean EC3 values and associated ranges will be obtained.

Dr. Basketter concluded by stating that ECVAM will not include more false positives and false negatives in its list. It has included one false positive and false negative in order to harmonize with ICCVAM but they don't see an added statistical value of just having one more false positive and false negative.

Karen Hamernik, EPA

Dr. Hamernik concurred with the comments that Dr. Rispin made previously, that performance standards, if developed such that they are too generalized with respect to minor versus major changes, would be problematic for regulatory agencies when they are reviewing submissions that include data from a modified LLNA protocol. Dr. Hamernik also asked for clarification from the Panel on a statement made during their discussions that a test for concordance for measuring the accuracy of classification (i.e., yes/no answer) should be done and that a chemical-for-chemical match is not necessary. Dr. Flournoy responded that concordance is not absolute but a continuum. Dr. Luster further clarified that the Panel discussion was based on the fact that the traditional LLNA is not a perfect match when compared to the guinea pig tests. Because there are false negatives and false positives compared to the guinea pig, there should be some flexibility so that an absolute chemical-by-chemical match is not required. In addition, a scientifically valid explanation can be provided for any discordance. Dr. Stokes emphasized that this was an important point and that additional clarity on the differences between a chemical-by-chemical match and overall accuracy need to be carefully considered before the final test method accuracy requirements are defined.

Panel Conclusions and Recommendations:

Dr. Luster asked the Panel to review the conclusions and recommendations for the ICCVAM LLNA performance standards they had discussed earlier and to make any revisions, if necessary. The Panel indicated that modified LLNA protocols that are undergoing validation should contain essential test method components that follow the ICCVAM-recommended protocol,[33] unless adequate scientific rationale for deviating from this protocol was provided. The Panel also identified aspects of the LLNA that should be required as part of the test method validation process, if more extensive changes to the protocol are being considered: (1) application of the test substance to the skin with sampling of the lymph nodes draining that site, (2) measurement of cell proliferation in the draining lymph node, (3) absence of a skin reaction that could be indicative of the onset of the elicitation phase of skin sensitization, (4) data collected at the level of the individual animal to allow for an estimate of the

[33] http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/LLNAProt.pdf

variance within control and treatment groups,[34] and (5) if dose response information is needed, there are an adequate number of dose groups ($n \geq 3$) with which to accurately characterize the dose response for a given test substance.

The Panel also recommended that statistical tests to analyze the data might allow for a more accurate interpretation. They recommended that a suitable variance-stabilizing transformation (e.g., log transformation, square root transformation) be applied in all statistical analyses and in reporting summary standard deviations. The Panel also recommended that a more rigorous evaluation be conducted of what would be considered an appropriate range of ECt values (i.e., estimated concentration needed to produce a stimulation index that is indicative of a positive response) to include as a requirement. This would be a statistical evaluation that considers the variability of ECt values generated among the sensitizers included on the performance standards reference substances list and the statistical multiple comparisons problem.

Dr. Luster asked the Panel if they agreed with the changes and revisions made at this point and with the Panel conclusions and recommendations as presented and revised. The members of the Panel agreed with one abstention; Dr. McDougal abstained from voting stating that he still had a concern about what constitutes a "major/minor" change. The Panel's detailed recommendations and conclusions on the ICCVAM LLNA performance standards are included in their final Panel report.[35]

Overview of the Draft LLNA Potency Determinations BRD and Draft ICCVAM Test Method Recommendations

Dr. Strickland presented an overview of the draft ICCVAM BRD for the use of the LLNA to determine skin-sensitization potency. She mentioned that the draft ICCVAM BRD provided a comprehensive review of the available data and information regarding the usefulness and limitations of the LLNA as a stand-alone assay for hazard categorization of skin-sensitization potency. In the BRD, the LLNA was evaluated for its ability to categorize substances for skin-sensitization potency using EC3 values.

Dr. Strickland noted that the analyses conducted in the BRD were based on LLNA studies obtained from ICCVAM (1999), the published literature, and data received in response to an FR notice (72 FR 27815, May 17, 2007) requesting original data from the LLNA. As a result, the analyzed data included 170 substances with LLNA, human, and/or guinea pig data. Dr. Strickland noted that three sets of data were analyzed and briefly summarized the results which are detailed in the draft ICCVAM BRD.[36] Dr. Strickland also briefly summarized the draft ICCVAM test method recommendations for potency determinations.[37]

Panel Evaluation:

Ms. Headrick presented her Evaluation Group's draft responses to ICCVAM's questions to the Panel for consideration by the entire Panel. These included their review of the draft BRD for errors and omissions, their overall assessment of the validation status of the test method, and their comments on the draft ICCVAM test method recommendations. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD and recommendations are reflected in the Panel report published in May 2008.[38]

During the course of the discussion on the potency applicability of the LLNA, Dr. Woolhiser asked what the basis for the human threshold concentration cutoff values of 250 and 500 $\mu g/cm^2$ were. Dr.

[34] Individual animal data will allow the application of a formal statistical test, if deemed necessary, and will also allow power calculations associated with the modified LLNA test.
[35] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[36] http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-pot/LLNApotency18Jan08FD.pdf
[37] http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-pot/LLNAPotencyRecs18Jan08FD.pdf
[38] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

Wind replied that a number of experts and clinicians from throughout the world went back and looked at what, in their countries, they demarcated as strong sensitizers. The proposed Globally Harmonized System of Classification and Labeling of Chemicals (GHS) subcategory guidance values for the LLNA, guinea pig tests (GPMT, BT) and human data (HMT and HRIPT) were made on the basis of an impact analysis of 175 chemicals. In addition, the two proposed cut-offs were evaluated by the GHS Expert Group on Sensitization based upon chemicals already regulated as strong sensitizers to ensure their inclusion within the GHS categorization scheme. Clinical members of the Expert Group also confirmed relevance of the cut-off values such that clinically important skin sensitizers fell into the appropriate subcategory. The proposed guidance values were also in line with the European Commission's Expert Working Group recommendations.

Public Comments:

Dr. David Basketter, ECVAM Observer
Dr. Basketter commented that reviewing the potency data by splitting it into pooled and unpooled groups could be interesting but might be difficult since the majority of available data likely comes from pooled groups. Furthermore, much of the deliberation concluding that individual animal data must be used was derived from analyses based only or largely on pooled data from four animals.

Dr. Basketter further stated that he viewed the analyses, which make the assumption that the human threshold data is the gold standard, as fundamentally flawed. Human data comes from studies conducted at different times, with different protocols, according to varying quality standards, and by different people. Therefore, there is no definitive knowledge of the reproducibility of the data. However, he considers the analyses adequate for recommending the LLNA as a part of a weight-of-evidence decision on human sensitization potency categorizations.

Dr. Amy Rispin, EPA
Dr. Rispin noted that there has been much discussion about various ways of handling the potency data. The OECD expert task force on skin sensitization needs to see an analytical comparison of what is considered to be the most appropriate approach for evaluating the data. The question for categorization purposes is, *What is the ideal testing modality for separating strong versus weak sensitizers for potency categorization*? A regulator who must assign a categorization is going to be confronted with all available test data and must know which data should be given the greatest weight in their evaluation.

Dr. Rispin noted that the OECD task force also reviewed the draft BRD on potency determinations and sent a list of several questions to the Panel, some of which have been answered, many of which have not been. One of the questions is, can the LLNA protocols be refined (e.g., by selection of solvents or choice of other test parameters) to improve correlation? She concluded by noting that she hopes that the additional analyses that the Panel has suggested will bring some clarity to the matter.

Panel Conclusions and Recommendations:

Dr. Luster asked the Panel to review the conclusions and recommendations for the LLNA potency determinations they had discussed earlier and to make any revisions, if necessary. The Panel agreed with the draft ICCVAM recommendation that the LLNA should not be used as a stand-alone assay for categorizing skin sensitizers as strong versus weak, but that it could be used as part of a weight-of-evidence evaluation (e.g., along with quantitative structure-activity relationships, peptide reactivity, human evidence, historical data from other experimental animal studies) for this purpose. The Panel also agreed with ICCVAM's recommendation that any LLNA studies conducted for the purpose of evaluating skin-sensitization potency should use the ICCVAM-recommended LLNA protocol. In addition, the Panel stated that the relevant testing guidelines for the traditional LLNA should be revised to include the procedure for calculating an EC3 value. Dr. Luster asked the Panel if they agreed with the changes and revisions made at this point and with the Panel conclusions and recommendations as presented and revised; the Panel unanimously agreed. The Panel's detailed

recommendations and conclusions on the LLNA potency determinations are included in their final Panel report.[39]

Concluding Remarks—

Dr. Luster, on behalf of the Panel, thanked the NICEATM-ICCVAM staff for their continued assistance during the review process and the Panel meeting. He also thanked Drs. Joanna Matheson and Abby Jacobs, the IWG co-chairs, and Dr. Marilyn Wind, ICCVAM Chair and IWG member, for the hard work they put into the project. Dr. Luster also thanked the Panel and the Panel Chairs for their involvement in the huge task of reviewing seven topics. He commented that, for future reference for ICCVAM, the Panel in their individual groups were able to do a good job in reviewing the materials, but because they were so focused on their particular topics due to serious time constraints, there may not have been the full benefit of their expertise for other topics in all cases.
Drs. Wind and Stokes thanked the Panel again for their hard work, thoughtful and objective deliberations, and advice. Dr. Stokes further thanked the invited test method developers for their excellent summaries of their method for the benefit of the Panel, and CPSC for hosting the Panel meeting. He mentioned that there has been discussion about obtaining additional existing data (i.e., on mixtures, on one or more of the non-radiolabeled test methods), and that should these data become available in a timely manner and if NICEATM is able to assimilate and analyze the data, the Panel might be reconvened by teleconference to review the data. Dr. Stokes concluded by saying he looked forward to further working with the Panel members to complete their Panel report.

Adjournment—

The meeting was adjourned and concluded at 3:20 p.m.

[39] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

William S. Stokes, D.V.M.
NIEHS
P.O. Box 12233
MD-EC17
Research Triangle Park, NC 27709

Dear Dr. Stokes,

The Meeting Summary Minutes, Independent Scientific Peer Review Panel Meeting, Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products, accurately summarizes the Peer Review Panel meeting of March 4-6, 2008, in Bethesda, MD.

Sincerely,

Signature	Printed Name	Date
	MICHAEL I LUSTER	11-18-08

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Appendix D2

Peer Review Panel Report: Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products

The full document is available electronically on the enclosed CD-ROM or at: http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

The document is also available on request from NICEATM:

NICEATM
National Institute of Environmental Health Sciences
P.O. Box 1233, MD K2-16
Research Triangle Park, NC 27709 USA
Telephone: 919-541-2384 Fax: 919-541-0947
E-mail: niceatm@niehs.nih.gov

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Independent Scientific Peer Review Panel Report: Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products

May 2008

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

National Institute of Environmental Health Sciences (NIEHS)
National Institutes of Health
U.S. Public Health Service
Department of Health and Human Services

National Toxicology Program
P.O. Box 12233
Research Triangle Park, NC 27709

This document is available electronically at:
http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

The findings and conclusions of this report are those of the Independent Scientific Peer Review Panel and should not be construed to represent the official views of ICCVAM or its member agencies.

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List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
ANOVA	Analysis of variance
AOO	Acetone: olive oil (4:1)
BRD	Background Review Document
BrdU	Bromodeoxyuridine
BT	Buehler Test
CD4	Cluster of differentiation 4
CPSC	U.S. Consumer Product Safety Commission
CRO	Clinical research organization
CV	Coefficient of variation
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNCB	Dinitrochlorobenzene
EC3	Estimated concentration needed to produce a stimulation index of three
ECt	Estimated concentration needed to produce a stimulation index that is indicative of a positive response
ECVAM	European Centre for the Validation of Alternative Methods
ELISA	Enzyme Linked Immunosorbent Assay
eLLNA: BrdU-FC	Enhanced LLNA with BrdU detected by flow cytometry
EPA	U.S. Environmental Protection Agency
FC	Flow cytometry
FR	*Federal Register*
GLP	Good Laboratory Practice
GPMT	Guinea Pig Maximization Test
GSK	GlaxoSmithKline
HCA	Hexyl cinnamic aldehyde
HMT	Human Maximization Test
HRIPT	Human Repeat Insult Patch Test
HTdR	^{3}H-Methyl Thymidine
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ISO	International Organization for Standardization
IWG	Immunotoxicity Working Group
JaCVAM	Japanese Center for Validation of Alternative Methods
LLNA	Local Lymph Node Assay
LLNA: BrdU-ELISA	LLNA with BrdU detected by ELISA
LLNA: BrdU-FC	LLNA with BrdU detected by FC
LLNA: DA	LLNA: Daicel Adenosine Triphosphate
LNC	Lymph node cells
LOEL	Lowest observed effect level
MEK	Methyl ethyl ketone
NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods

NIEHS	National Institute of Environmental Health Sciences
NIH	National Institutes of Health
NOEL	No observed effect level
NTP	National Toxicology Program
OD	Optical density
OECD	Organisation for Economic Co-operation and Development
QSAR	Quantitative structure-activity relationship
REACH	Registration, Evaluation, and Authorisation of Chemicals
rLLNA	Reduced LLNA
SAR	Structure-activity relationship
SD	Standard deviation
SI	Stimulation index
SDS	Sodium dodecyl sulfate
SLS	Sodium lauryl sulfate
TG	Test Guideline
Th	T-helper
vs.	Versus

Members of the Independent Scientific Peer Review Panel

Michael Luster, Ph.D. (Panel Chair), Senior Consultant to the National Institute of Occupational Safety and Health (NIOSH) Health Effects Laboratory, Morgantown, WV

Nathalie Alépée, Ph.D., Associate Research Fellow, Pfizer PDRD MCT Laboratory, France

Anne Marie Api, Ph.D., Vice President, Human Health Sciences, Research Institute for Fragrance Materials, Woodcliff Lake, NJ

Nancy Flournoy, M.S., Ph.D. (Biostatistician), Professor and Chair, Dept. of Mathematics & Statistics, University of Missouri-Columbia, Columbia, MO

Thomas Gebel, Ph.D., Regulatory Toxicologist, Federal Institute for Occupational Safety & Health, Dortmund, Germany

Sidney Green, Ph.D.,[1] Graduate Professor, Howard University, Washington, DC

Kim Headrick, B. Admin., B.Sc., International Harmonization Senior Policy Advisor, Health Canada, Ottawa, Ontario, Canada

Dagmar Jírová, M.D., Ph.D., Toxicologist, Research Manager, Head of Reference Center for Cosmetics, Head of Reference Laboratory for Experimental Immunotoxicology, National Institute of Public Health, Czech Republic

David Lovell, Ph.D. (Biostatistician), Reader in Medical Statistics, Postgraduate Medical School, University of Surrey, Guildford, Surrey, U.K.

Howard Maibach, M.D., Professor, Department of Dermatology, University of California-San Francisco, San Francisco, CA

James McDougal, Ph.D., Professor and Director of Toxicology Research, Department of Pharmacology and Toxicology, Boonshoft School of Medicine, Wright State University, Dayton, OH

Michael Olson, Ph.D., Director of Occupational Toxicology, Corporate Environment Health and Safety, GlaxoSmithKline, Research Triangle Park, NC

Raymond Pieters, Ph.D., Associate Professor, Immunotoxicology Group Leader, Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands

Jean Regal, Ph.D., Professor, Department of Pharmacology, University of Minnesota Medical School, Duluth, MN

Jonathan Richmond, MB ChB, FRCSEd,[1] Head of Division, Animals Scientific Procedures Division, Home Office, London, U.K.

Peter Theran, V.M.D., Massachusetts Society for the Prevention of Cruelty to Animals, Novato, CA

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[1] Drs. Green and Richmond were unable to attend the public meeting on March 4-6, 2008. However, they were involved in the review of the background review documents and concur with the conclusions and recommendations included in this report.

Stephen Ullrich, Ph.D., Dallas/Ft. Worth Living Legends Professor & Professor of Immunology, Post-graduate School of Biomedical Science, University of Texas M.D. Anderson Cancer Center, Houston, TX

Michael Woolhiser, Ph.D., Technical Leader - Immunotoxicology, Toxicology & Environmental Research & Consulting Immunology, Dow Chemical, Midland, MI

Takahiko Yoshida, M.D., Ph.D., Professor, Department of Health Science, Asahikawa Medical College, Hokkaido, Japan

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Preface

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended the murine local lymph node assay (LLNA) to U.S. Federal agencies as a valid substitute for currently accepted guinea pig test methods to assess the allergic contact dermatitis potential of many, but not all, types of substances. The recommendation was based on a comprehensive evaluation of the validation status of the LLNA that included an assessment by an international independent scientific peer review panel (hereafter, Panel). The Panel report and the ICCVAM LLNA test method recommendations (ICCVAM 1999) are available at the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)-ICCVAM website.[1] The LLNA was subsequently incorporated into national and international test guidelines for the assessment of skin sensitization (OECD 2002; ISO 2002; EPA 2003). For this Panel report, this LLNA will be referred to as the "traditional" LLNA.

On January 10, 2007, the U.S. Consumer Product Safety Commission (CPSC) formally requested through NICEATM that ICCVAM assess the validation status of:[2]

- The traditional LLNA as a stand-alone assay for potency determinations (including severity) for the purpose of hazard classification
- Three modifications of the traditional LLNA not requiring the use of radioactive materials
- The LLNA limit dose procedure (also referred to as the "reduced" LLNA)
- The ability of the traditional LLNA to test mixtures, metals, and aqueous solutions (i.e., to re-evaluate the applicability domain for the traditional LLNA)

NICEATM, in coordination with ICCVAM and the ICCVAM Immunotoxicity Working Group, prepared a comprehensive draft background review document (BRD) for each modified version of the traditional LLNA test method being evaluated, as well as a draft applicability domain addendum to the final BRD published previously on the traditional LLNA. Each draft BRD and the draft addendum detailed the available data and information from the published literature and submissions received in response to a 2007 *Federal Register (FR)* notice that had requested data related to CPSC's nomination (*FR* notice Vol. 72, No. 95, p. 27815-27817, May 17, 2007). In addition, ICCVAM developed draft LLNA Performance Standards intended for use in validating alternative test methods that are functionally and mechanistically similar to the traditional LLNA. Finally, ICCVAM, based on the information contained in each of the draft BRDs and the draft addendum, developed draft test method recommendations.

The various supporting documents and the draft ICCVAM recommendations were provided to a new international Panel for an independent scientific review. In addition, NICEATM announced the availability of these documents on the NICEATM-ICCVAM website

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[1] The 1999 ICCVAM Panel report and recommendations can be obtained at: http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf

[2] The CPSC nomination can be obtained at: http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf

(http://iccvam.niehs.gov) for public comment in a *FR* notice (Vol. 73, No. 5, p. 1360-1362, January 8, 2008) and via the ICCVAM listserv. The *FR* notice also announced the public Panel meeting, to be convened at the CPSC Headquarters in Bethesda, MD on March 4–6, 2008.

The Panel was charged with:

- Reviewing each ICCVAM draft BRD and the draft addendum for completeness and identifying any errors or omissions of existing relevant data or information
- Evaluating the information in each draft BRD and the draft addendum to determine the extent to which each of the applicable criteria for validation and acceptance of toxicological test methods (ICCVAM 2003) had been appropriately addressed for the recommended use of the new versions and applications of the traditional LLNA
- Considering the ICCVAM draft test method recommendations for the following and commenting on the extent to which they are supported by the information provided in the draft BRDs and the draft addendum:
  - proposed test method uses
  - proposed recommended standardized protocols
  - proposed test method performance standards
  - proposed additional studies
- Evaluating the draft ICCVAM LLNA Performance Standards and considering whether they were adequate for assessing the accuracy and reliability of alternative test methods that are functionally and mechanistically similar to the traditional LLNA

During our public meeting in March 2008, the Panel discussed each charge, listened to public comments, and developed conclusions and recommendations for ICCVAM on each of the nominated activities. The Panel wished to emphasize that they were to consider two overall questions. They were to consider: (1) whether the validation status of the each of the above proposed modifications or alternative uses of the LLNA had been adequately characterized for its intended purpose according to established ICCVAM validation criteria (available on the NICEATM-ICCVAM website, http://iccvam.niehs.gov), and (2) whether proposed modifications or alternative uses of the LLNA are sufficiently accurate and reliable to be used for the identification of sensitizing substances and non-sensitizing substances in place of the traditional LLNA procedure.

This report details the Panel's independent conclusions and recommendations. ICCVAM will consider this report, along with all relevant public comments, as it develops final test method recommendations. The final ICCVAM test method recommendations will be forwarded to U.S. Federal agencies for their consideration in accordance with the ICCVAM Authorization Act of 2000 (Public Law 106-545).

The Panel gratefully acknowledges the efforts of NICEATM staff in coordinating the logistics of the peer review Panel meeting and in preparing materials for their review. The

Panel also thanks each of the test method developers, Drs. George DeGeorge (LLNA: BrdU-FC), Kenji Idehara (LLNA: DA), and Masahiro Takeyoshi, (LLNA: BrdU-ELISA) for providing summaries and additional clarifications of the non-radioactive test methods under review. Finally, as Panel Chair, I want to thank each Panel member for her or his thoughtful and objective review of these LLNA-related activities.

Michael Luster, Ph.D.
Chair, LLNA Peer Review Panel
May 2008

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Executive Summary

This report describes the conclusions and recommendations of an international independent scientific peer review panel (hereafter, Panel). This Panel was charged by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) with evaluating the validation status of new versions and applications of the murine local lymph node assay (LLNA) for assessing the allergic contact dermatitis (ACD) potential of chemicals and products. The LLNA, which was first evaluated in 1999 by ICCVAM, is hereafter referred to as the "traditional LLNA" to distinguish it from other versions considered by the Panel. The new versions and applications considered include:

- The LLNA limit dose procedure (also referred to as the "reduced" LLNA[1])
- The ability of the traditional LLNA to test mixtures, metals, and aqueous solutions (i.e., a re-evaluation of the applicability domain for the traditional LLNA)
- Three modifications of the traditional LLNA not requiring the use of radioactive materials:
  - LLNA: DA (Local Lymph Node Assay: Daicel Adenosine Triphosphate)
  - LLNA: BrdU-FC (Local Lymph Node Assay: Bromodeoxyuridine detected by flow cytometry)
  - LLNA: BrdU-ELISA (Local Lymph Node Assay: Bromodeoxyuridine detected by ELISA)
- The traditional LLNA as a stand-alone assay for potency determinations (including severity) for the purpose of hazard classification

The Panel also evaluated the draft ICCVAM LLNA Performance Standards and considered whether they were adequate for assessing the accuracy and reliability of alternative test methods that are functionally and mechanistically similar to the traditional LLNA.

LLNA Limit Dose Procedure

The Panel agreed that the LLNA limit dose procedure, which normally allows for testing at one dose level, should be routinely recommended for hazard identification when used for testing purposes which do not require dose response information, because it would offer time, cost, throughput and logistical benefits as well as using fewer animals. In instances when a necessity to measure relative skin sensitization potency for the purpose of risk assessment was present, then the traditional LLNA should be used in order to generate dose response information. Still, the Panel recommended use of the LLNA limit dose procedure as

[1] As described in this report, the Panel agreed that consideration should be given to applying the same term to the LLNA limit dose procedure since in various places throughout the draft BRD it was referred to differently as either the "cut-down", the "limit dose", or the "reduced LLNA" (i.e., "rLLNA"). Since the European Centre for the Validation of Alternative Methods (ECVAM) has already established a naming convention of "rLLNA", the Panel recommended adopting the ECVAM terminology to harmonize the terminology used among the international validation agencies. However, because the ICCVAM documents that were reviewed use "LLNA limit dose procedure" that term is retained in this report.

the initial testing procedure to identify sensitizers and non-sensitizers before conducting the traditional LLNA even when dose response information *is* required since if the test substance were negative in the limit dose procedure, it would not be necessary to conduct a multiple-dose LLNA test.

The draft background review document (BRD) for the LLNA limit dose procedure provides a comprehensive review of available data and information for assessing the usefulness and limitations of this modified version of the LLNA for the purpose of skin sensitization hazard classification. The Panel evaluated the draft BRD for completeness, errors, and omissions and recommended that it be updated to reflect their suggestions/corrections relating to general, statistical, and specific editorial issues. In particular, the Panel noted that the differences in terminology used for this procedure caused confusion and recommended that an internationally harmonized term be adopted. They suggested referring to the procedure as the "reduced LLNA" (i.e. "rLLNA") since that is being used by the European Centre for the Validation of Alternative Methods (ECVAM).

The Panel concluded that the stimulation index (SI) based on the ratio of 3.0 as the cutoff value was indicative of a response that was sufficiently greater than the control and would be considered an immunologically relevant response, but recommended that statistical analyses be used to definitively establish that a response induced by a test substance is significantly different from the vehicle control. The Panel agreed that the LLNA protocol recommended by ICCVAM (ICCVAM 1999; Dean et al. 2001) should be the standard protocol for all future LLNA limit dose studies using the traditional LLNA protocol. Specifically, prospective LLNA limit dose procedure studies should require that lymph nodes be collected from individual animals instead of pooling them with other animals in a treatment group, which is also currently permitted by the Organisation for Economic Co-operation and Development (OECD) Test Guideline 429 (OECD 2002). Individual animal response data are necessary in order to statistically analyze for differences between treated and control data. In addition, having data from individual animals also allows for identification of technical problems and outlier animals within a dose group. Based on power calculations provided as supplemental information, the Panel agreed that five animals per dose group is an appropriate number to recommend for LLNA limit dose studies following the traditional LLNA protocol. It should be noted that the Panel's analysis of the LLNA limit dose dataset was not restricted to studies with confirmed individual animal data, and that the Panel considered data known to have been generated using pooled group data. The Panel stated that, internationally, both individual and pooled animal data have likely been used both for regulatory decisions and for in-house decisions relating to product development and risk management. In addition, the fact that the retrospective data analysis set out in the draft LLNA limit dose procedure BRD did not distinguish between individual or pooled animal data suggested that both met the quality standards for inclusion in the draft BRD.

Although they did not reach consensus, the Panel suggested that for laboratories in which the LLNA is "routinely" performed and have demonstrated the ability to consistently obtain positive results, hexyl cinnamic aldehyde (HCA) or another positive control (e.g., a substance that matches the chemical class of the test substances) could be run at intervals for quality control purposes rather than concurrent with each experiment. The Panel cited Kimber et al. (2006), which describes "routine" use of the "rLLNA" utilizing only a vehicle and a high-dose group, as a rationale for this suggestion. However, the Panel does not recommend

omitting the concurrent positive control in laboratories that perform the LLNA only "occasionally".

Based on the analyses presented in the draft BRD, the Panel considered the accuracy of the LLNA limit dose procedure to have been adequately evaluated and compared to the traditional LLNA, mindful of the limitations associated with a retrospective evaluation. For instance, it cannot be assumed that the compounds tested in the retrospective studies were always tested at the highest possible dose unless such information was explicitly indicated. In this regard, the Panel recommended that a more detailed description of what is considered "*avoidance of excessive irritation*" and "*evidence of systemic toxicity*" be included in any LLNA protocol in order to aid in choosing the most appropriate high (i.e., limit) dose, although specific indicators of "*systemic toxicity or excessive irritation*" were not formally discussed.

The Panel agreed that it was appropriate to assume that the intra- and inter-laboratory reproducibility of the LLNA limit dose procedure and the traditional LLNA would be similar, because reproducibility is more dependent on the method than on the number of dose groups. However, reducing the number of test substances dose groups from three to one might reduce the sensitivity of the assay. The traditional LLNA may have a greater chance of correctly identifying a sensitizer even in the presence of one or more technical errors since data from three dose groups are being considered and an SI ≥3.0 at any dose group would result in the substance being classified as a sensitizer. However, for the purpose of adopting an assay that uses fewer animals and provides increased throughput for testing purposes, these hypothetical considerations are not a sufficient reason to argue against use of the limit dose LLNA procedure.

LLNA for Testing Aqueous Solutions, Metals, and Mixtures

The draft ICCVAM recommendations state that, although more data are needed to assess the use of the LLNA for testing for mixtures and aqueous solutions before a recommendation can be made, the traditional LLNA appears to be useful for the testing of metal compounds, with the exception of nickel. The Panel agreed with these draft ICCVAM recommendations. Regarding the use of the LLNA for testing mixtures, the Panel acknowledged that the ability of ICCVAM to develop draft test method recommendations was limited not only by the amount of data available, but the relatively poor concordance of traditional LLNA outcomes in comparison to those obtained in guinea pig tests, and recommended that this be noted in the final ICCVAM recommendations. The term "mixtures" can represent an infinite number of materials and it would be more beneficial to specify types or formulations of mixtures that are being examined.

Regarding metals, the Panel concluded that the accuracy statistics for the traditional LLNA when compared to results obtained from evaluation in humans supported use of the traditional LLNA as a hazard identification tool for metals, with the exception of nickel, which produces variable responses. One minority opinion stated that the results for nickel compounds were not entirely questionable and that the traditional LLNA might also be suitable for testing nickel compounds. Thus, the Panel recommended further evaluation of the variable results obtained for nickel in the context of the available literature on allergic contact dermatitis to nickel in humans.

Regarding substances tested in aqueous solutions, the Panel suggested expanding the brief section of the draft test method recommendations discussing the test method protocol for the traditional LLNA to specifically point out how the conclusions of the applicability domain evaluation may affect the standard traditional LLNA protocol. For instance, it could be suggested that aqueous test solutions be avoided due to problems associated with skin application. It would be preferable for a hierarchy of organic solvents to be considered as dosing vehicles, with emphasis on using a vehicle to which humans may actually be exposed in circumstances linked to occupational sensitization.

The Panel agreed with the draft ICCVAM recommendation for continued accrual of information from traditional LLNA evaluations of mixtures, metals, and aqueous solutions with comparative data for guinea pig (i.e., guinea pig maximization test [GPMT] or Buehler test [BT]) and human (i.e., human maximization test [HMT] or human repeat insult patch test [HRIPT]) tests. However, the Panel suggested that, given resource limitations, it would be important to organize the recommendations based on relative priority.

The draft Addendum to the original validation report for the traditional LLNA (ICCVAM 1999) provided a comprehensive review of currently available data and information for evaluating the usefulness and limitations of the traditional LLNA for assessing the skin sensitization potential of mixtures, metal compounds, and substances tested in aqueous solutions. The Panel evaluated the draft Addendum for completeness, errors, and omissions and concluded that there were no apparent errors or omissions, although they did state that the term “mixtures” was used too broadly (i.e., can represent an infinite number of materials) and it would be more beneficial to specify types or formulations of mixtures that are being examined.

The Panel did not identify any classes of chemicals missing from the dataset used to review the utility of the traditional LLNA for testing aqueous solutions. However, while they did not propose an alternative, the Panel expressed concern over the most appropriate definition for an aqueous solution (defined in the draft Addendum as any solution containing ≥20% water). For the mixtures included in the analysis, the Panel noted that quantitative compositions had not been provided and therefore they could not comment on whether these mixtures were representative of the types of mixtures typically tested in the traditional LLNA. With respect to metals (none of which are mixtures), there was a paucity of important representatives of commercially useful metals such as platinum, palladium, iron, zinc, manganese and silver in the data set. The Panel suggested that to enlarge the group of metal non-sensitizers, substances used as cosmetic ingredients (e.g., titanium dioxide) and aluminum compounds currently used in antiperspirants might be considered.

The Panel agreed that, although it was important to identify data obtained according to GLP guidelines, data obtained from non-GLP studies should not be excluded automatically from this retrospective analysis. The Panel concluded that other factors could be used to identify high quality data. Examples include data published in peer-reviewed journals or obtained from a study conducted in a laboratory that has GLP capabilities.

The Panel concluded that, considering the limited comparative data that were available, particularly for mixtures and aqueous solutions, the accuracy assessment of the traditional LLNA for testing mixtures, metals, and aqueous solutions when compared to available human and/or guinea pig test results was as comprehensive as was possible. The limited

amount of comparative data made it unfeasible to draw definitive conclusions for mixtures and aqueous solutions from the available accuracy statistics.

Non-Radioactive LLNA Protocol - The LLNA: DA Test Method

The Panel concluded that the available data and test method performance support the ICCVAM draft recommendations for the LLNA: Daicel Adenosine Triphosphate test method (LLNA: DA), and that the test method may be useful for identifying substances as potential skin sensitizers and non-sensitizers, but that this recommendation is contingent upon receipt, review, and analyses of additional existing data and information from the test method developer. Therefore, this non-radioactive version of the traditional LLNA cannot currently be recommended for the hazard identification of skin sensitizing substances, regardless of whether or not there are restrictions on the use of radioactive materials, until such time as this existing data has been received and confirmed.

The draft LLNA: DA BRD was compiled to provide a comprehensive review of available data and information evaluating the usefulness and limitations of the LLNA: DA test method to assess the allergic contact dermatitis potential of chemicals and other substances. The Panel evaluated the draft BRD for completeness, errors, and omissions and recommended that their suggestions/corrections relating to general, statistical, and specific editorial issues be incorporated into future revisions.

The Panel agreed that five animals per dose group should be recommended for validation of modified LLNA test methods. The Panel, however, noted that supplemental power calculations for the LLNA: DA test method indicated that the power for detecting a three-fold increase in the treatment group was estimated to be 95% for a sample size of three mice per dose group. Thus, the Panel identified the use of three animals per dose group as a potential opportunity to reduce animal number when using modified assays in the future, assuming all essential validation requirements can be successfully met. A minority opinion expressed by five Panel members was that if laboratories were operating under OECD guidance (OECD 2002) and a reliable validation dataset had been generated, then pooled data from a least four animals per dose group could be considered.

Generally, the Panel viewed the difference in treatment schedule between the LLNA: DA and the traditional LLNA to be potentially significant if the LLNA: DA induced the elicitation phase of skin sensitization. The Panel was concerned that the 1% sodium lauryl sulfate (SLS) pretreatment step in the LLNA: DA might modify the inherent sensitivity of the LLNA. They recommended that the test method developer (Daicel Chemical Industries, Ltd.) justify the use of 1% SLS or consider an alternative decision criterion (i.e., an SI threshold other than 3.0) such that the 1% SLS pretreatment is no longer necessary.

The Panel considered the database of substances tested in the LLNA: DA to be representative of a sufficient range of chemicals expected to be tested for skin sensitization potential, and concluded that the accuracy analysis had made appropriate comparisons to the traditional LLNA, guinea pig tests, and human data/experience. The Panel could not identify specific characteristics associated with the one false negative (i.e., 2-mercaptobenzothiazole) or the one false positive (i.e., benzalkonium chloride), but reemphasized that the potential impact of pretreatment with 1% SLS in this context needed to be considered.

With regard to test method reliability, the Panel concluded the intralaboratory reproducibility of the LLNA: DA had not been adequately evaluated. They noted that the two sensitizers tested had similar chemical structures (i.e., eugenol and isoeugenol) and that it was unclear if the tests were truly independent. The Panel also noted that the interlaboratory reproducibility of the assay could not be adequately evaluated given the lack of original laboratory data and limitations in the study design. In particular, they cited the use of pooled lymph nodes from the mice in each treatment group and the testing of each substance at predetermined dose levels established by the lead laboratory as study design limitations. Still, a Panel minority considered pooled data acceptable and the setting of dose levels for all laboratories based on results from the lead laboratory to be reasonable.

The Panel also commented that ideally, test substances should be coded during the validation of a new assay, although they did not feel that a lack of coding constituted a reason for rejecting the current LLNA: DA dataset. The Panel also commented that although GLP compliance is highly recommended for validation studies, the current studies should not be rejected solely on the basis of a lack of GLP compliance. However, the Panel considered it important to obtain the original records for all validation studies (which have been requested by NICEATM) in order to confirm that the reported data were the same as the data recorded in the laboratory notebooks.

With regard to the 5% (1/19) false negative and 10% (1/10) false positive rates obtained with the LLNA: DA, the Panel commented that it was important to identify reasons why the substances gave “false” results, taking into consideration factors such as intended use of the substances and the target population. They agreed that it might be useful to follow a suspected inaccuracy with an investigation of the mechanistic basis for the discordance since it may help to establish a biologically-based rationale for the discordance.

The Panel noted that the available LLNA: DA data did not support all of the ICCVAM draft recommendations in the proposed test method standardized LLNA: DA protocol. First, although the Panel agreed with the ICCVAM protocol that recommends five animals per dose group, they noted that supplemental statistical information provided for the LLNA: DA test method implied that using less than five animals per dose group was acceptable (e.g., a 3.0-fold increase in the SI value would likely be detected with 99% confidence when using four animals per dose group). In addition, the Panel considered it important to adequately characterize the effect of the 1% SLS pretreatment step in the LLNA: DA, and it should be demonstrated that the day 8 applications do not induce a skin reaction that could be indicative of the onset of the elicitation phase of skin sensitization. Keeping these points in mind, the Panel agreed that if the limit dose procedure was applicable to the traditional LLNA, then it would also be applicable to the LLNA: DA in order to further reduce the number of animals used.

The Panel also stated that the available data supported the ICCVAM draft recommendations for the LLNA: DA in terms of future studies, which included performing a more comprehensive evaluation using more non-sensitizers within and across laboratories. A minority opinion stated by one Panel member was that although testing more sensitizers might be warranted for interlaboratory validation studies, a sufficient number of non-sensitizers (n=11) had already been tested within the same laboratory.

The Panel also commented that the protocol differences between the LLNA: DA and the traditional LLNA could not clearly be constituted as "major" or "minor" changes. However, they considered this issue largely irrelevant if a test method was able to correctly predict the dermal sensitization potential of a test substance. Consequently, the Panel concluded that the current draft ICCVAM Performance Standards could be applicable to the LLNA: DA as a mechanistically and functionally similar test method.

Non-Radioactive LLNA Protocol - The LLNA: BrdU-FC Test Method

Overall, the Panel concluded that the available data and test method performance of the LLNA with bromodeoxyuridine (BrdU) detected by flow cytometry (LLNA: BrdU-FC) supported the draft ICCVAM recommendations that it may be useful for identifying substances as potential skin sensitizers and non-sensitizers, but that more information and existing data must be made available before the LLNA: BrdU-FC can be recommended for routine use. The Panel concluded that the test method usefulness and limitations identified in the draft ICCVAM recommendations accurately summarized the limits of the information supplied and the additional information that would need to be generated or provided for further consideration of the test method. As a result, the Panel concluded that the LLNA: BrdU-FC could not currently be considered as a scientifically valid replacement alternative to the traditional LLNA. Still, the Panel suggested that the test method recommendation should clearly state that the test method was not "invalid", but simply that there was currently not sufficient evidence and information to state that it had been adequately validated.

The draft LLNA: BrdU-FC BRD was compiled to provide a comprehensive review of available data and information evaluating the usefulness and limitations of the LLNA: BrdU-FC test method to assess the ACD-inducing potential of substances. The Panel evaluated the draft BRD for completeness, errors, and omissions and recommended that their recommendations/corrections relating to general, statistical, and specific editorial issues be incorporated into future revisions.

The LLNA: BrdU-FC included routine measurements of ear swelling as an indicator of excessive skin irritation. The Panel viewed that this, or any other quantitative measurement of skin irritation, should be carefully considered for inclusion in all LLNA protocols. The Panel considered inclusion of optional quantification of immunophenotypic markers as an additional mechanism for distinguishing irritants from sensitizers to be useful, as it might reduce the frequency of false positives (i.e., substances which are actually skin irritants) and improve comparisons with human data. However, they considered application of immunological markers too detailed and costly for routine LLNA use (i.e., for hazard classification purposes) and more suited for research purposes.

The Panel noted that the substances tested in the LLNA: BrdU-FC seemed representative of a sufficient range of chemical classes and physical chemical properties, and thus that the test method appeared applicable to many of the types of chemicals and products that are typically tested for skin sensitization potential. However, the Panel considered the total database available for evaluation of the validation status of the LLNA: BrdU-FC to be relatively small compared to the large number of substances assessed in the traditional LLNA. Therefore, the Panel recommended caution when making conclusions related to its concordance with the

traditional LLNA. Still, the accuracy of the LLNA: BrdU-FC was considered adequately evaluated and comparable to the traditional LLNA.

The Panel concluded that intralaboratory reproducibility was not adequately assessed and it should be better evaluated in order to support the validation of this test method. The Panel suggested that although the studies evaluated in the draft BRD were not GLP-compliant, this should not affect acceptance of the data for an evaluation of the validation status of this test method. However, some sources of variability in the intralaboratory data, such as failure to appreciate differences in composition of dosing solutions between experiments caused by test article instability or other phenomena, might be obscured if not in complete compliance with GLP guidelines. Thus, the Panel suggested that any additional studies undertaken to validate the test method should ideally be GLP-compliant.

The Panel agreed that the available data supported the ICCVAM draft recommendations for the LLNA: BrdU-FC procedure in terms of the proposed test method standardized protocol. They suggested that the utility of ear swelling or other methods to detect inflammation appeared warranted in every variation of the LLNA (including the traditional LLNA), but should be further investigated before routine inclusion in the protocol is recommended. The Panel also concluded that the traditional LLNA limit dose procedure could be applied to the LLNA: BrdU-FC, keeping in mind the limitations associated with a "limit dose" procedure.

The Panel further agreed that the ICCVAM draft recommendations for future studies highlighted the unanswered questions raised by the available data set. Specifically, conducting interlaboratory studies as a part of the validation process is important. The Panel considered the immunological markers suggested for the LLNA: BrdU-FC to be acceptable, but that additional immunological markers for discrimination of irritant versus sensitization phenomena were also possible. In general, for any future work, efforts should be made to decrease the variability and to thereby increase the power of the test in order to ensure that more animals were not needed relative to the traditional LLNA or other alternative LLNA protocols.

The Panel considered the protocol differences between the LLNA: BrdU-FC and the traditional LLNA to be "minor" changes, and therefore concluded that assessment of the validity of this test method could be based on the draft ICCVAM LLNA Performance Standards. The Panel also cautioned, however, that a clear definition of what constituted a "major" versus a "minor" change, or a different protocol altogether could be better addressed once the recommendations for the current draft ICCVAM LLNA Performance Standards were finalized.

Non-Radioactive LLNA Protocol - The LLNA: BrdU-ELISA Test Method

The Panel concluded that the available data and test method performance for the LLNA with BrdU detected by enzyme-linked immunosorbent assay (LLNA: BrdU-ELISA) support the ICCVAM draft recommendations that it may be useful for identifying substances as potential skin sensitizers and non-sensitizers, but that more information and existing data must be made available before the LLNA: BrdU-ELISA can be recommended for use. The Panel also stated that a detailed protocol was needed, in addition to sufficient quantitative data for broader analysis on a larger set of balanced reference substances that take into account

physicochemical properties and sensitization potency, as well as an appropriate evaluation of interlaboratory reproducibility.

The draft LLNA: BrdU-ELISA BRD was compiled to provide a comprehensive review of available data and information evaluating the usefulness and limitations of the LLNA: BrdU-ELISA test method to assess the ACD-inducing potential of chemicals and other substances. The Panel evaluated the draft BRD for completeness, errors, and omissions and recommended that their suggestions/corrections relating to general, statistical and specific editorial issues be incorporated into the final document.

The Panel's main concern with the test method was that the accuracy of the LLNA: BrdU-ELISA at SI ≥3.0 was inadequate and not equivalent to the traditional LLNA. Furthermore, although using a decision criterion of SI ≥1.3 improved the test's performance in identifying sensitizers from non-sensitizers, it did not resolve concerns about the test method. Based on a power analysis for the LLNA: BrdU-ELISA, which was provided to the Panel as supplemental information, the Panel concluded that it was difficult to justify using a SI ≥1.3 as the cutoff value, given the much larger number of animals that would be required to detect a 1.3-fold increase above vehicle controls with similar power to the traditional LLNA when five animals per dose group are used. For a three-fold increase, the supplemental statistical analyses indicated that a sample size of four was sufficient. Still, the Panel agreed with the ICCVAM recommendation to use five animals per dose group and to collect individual animal data. They concluded that this would allow for more robust calculations in the event that an outlier prevented some of the data from being included in the analysis. A minority opinion by five Panel members was stated that if laboratories were operating under OECD guidance (OECD 2002) and a reliable validation dataset had been generated, then pooled data from a least four animals could be considered.

The Panel noted that in organizations where the use or disposal of radioactive materials was restricted, the potential to use the LLNA: BrdU-ELISA could reduce the number of animals needed per test compared to the traditional LLNA and would result in less pain and suffering compared to using traditional guinea pig test methods. However, if the SI ≥1.3 was chosen as the decision criterion because of its improved accuracy compared to SI ≥3.0, the Panel stated that the number of mice needed to perform the LLNA: BrdU-ELISA test should be compared to the number of guinea pigs that would be needed for skin sensitization tests in order to assess if the LLNA: BrdU-ELISA actually reduced overall animal use for skin sensitization testing.

In general, the Panel considered the number of substances tested in the LLNA: BrdU-ELISA too few, and that data from more substances tested using the traditional LLNA, guinea pig tests, and human tests should have been included. The Panel also did not consider the available data from the LLNA: BrdU-ELISA to be representative of a sufficient range of chemical classes and physical chemical properties. The limited dataset prevents an evaluation of whether the test method would be considered applicable to any of the types of chemicals and products typically tested for skin sensitization potential.

However, the Panel concluded that the appropriate comparisons between the traditional LLNA, guinea pig test and human data had been made. The Panel agreed that the false negative rate for hazard identification using the SI ≥3.0 in the LLNA: BrdU-ELISA was excessive (i.e., using this SI threshold value, the LLNA: BrdU-ELISA misclassified 29% and

39% of the substances classified as sensitizers in the traditional LLNA or in humans, respectively).

The Panel also considered that the intralaboratory reproducibility of the LLNA: BrdU-ELISA was not adequately evaluated and compared to the traditional LLNA. The Panel indicated that the number of substances was too few, and in some cases there was a wide variation in repeat tests of the same substance. The Panel recommended a more comprehensive evaluation of the intralaboratory reproducibility of the test method, using different SI values, and that the analysis of the variability of the estimated concentration needed to produce a positive SI value (ECt values) be conducted on a log scale.

The Panel also noted that interlaboratory reproducibility for the LLNA: BrdU-ELISA could not be evaluated because neither the design of the study sponsored by the Japanese Center for Validation of Alternative Methods nor any of the resulting data had been provided in advance of their evaluation. The Panel agreed that a multi-laboratory validation study using a balanced set of chemicals would adequately characterize the interlaboratory reproducibility of the LLNA: BrdU-ELISA.

In general, the Panel agreed that the available data support the ICCVAM draft recommendations for the LLNA: BrdU-ELISA procedure in terms of the proposed test method standardized protocols. However, as noted above, a minority opinion by five Panel members was that there could be circumstances in which pooled data from at least four animals could also be acceptable. The Panel also stated that if the LLNA: BrdU-ELISA was found to be equivalent to the traditional LLNA in the future that it would be appropriate to apply the LLNA limit dose procedure to the test. The Panel also agreed with ICCVAM's test method recommendations for future studies and emphasized that more data were needed in order to determine the appropriate threshold value for the decision criterion. The Panel concluded that it might be more appropriate to use a statistically-based decision criterion rather than a stimulation index to classify substances as sensitizers, and that this should be further investigated.

The Panel agreed that the LLNA: BrdU-ELISA protocol differed from the traditional LLNA only in the method used to assess lymphocyte proliferation and as such concluded that this represented a "minor" change (as defined in the current draft ICCVAM LLNA Performance Standards) and separate performance standards for the LLNA: BrdU-ELISA were not needed.

Draft ICCVAM LLNA Performance Standards

The draft ICCVAM LLNA Performance Standards are intended to evaluate the acceptability of proposed test methods that are mechanistically and functionally similar to the traditional LLNA. ICCVAM proposed that the applicability of the draft ICCVAM LLNA Performance Standards be restricted to protocols that incorporate "minor" modifications to the traditional LLNA procedure, defined as changes only to the method for measuring lymphocyte proliferation. The Panel agreed that different methods of measuring lymphocyte proliferation represent "minor" modifications, but recommended that, instead of trying to define "minor" modifications, a better strategy might be to define criteria that would need to be satisfied in order to ensure that the alternative test method was mechanistically and functionally similar to the traditional LLNA (e.g., only measure cell proliferation associated with the induction

phase of a skin sensitization reaction). The Panel considered that the draft performance standards were also appropriate for evaluating other modifications. Examples of acceptable modifications included test animal sex, strain, the use of rats rather than mice, the number of animals per group, and timing of test article treatment. One minority opinion considered the potential impact of changes to protocol components other than the method of measuring lymphocyte proliferation to be significant and therefore would require more extensive validation, which was not defined.

The Panel indicated that alternative LLNA protocols that are undergoing validation should contain essential test method components that follow the ICCVAM-recommended protocol (ICCVAM 1999; Dean et al. 2001), unless adequate scientific rationale for deviating from this protocol was provided.

The Panel also identified aspects of the LLNA that should be required as part of the test method validation process: (1) application of the test substance to the skin with sampling of the lymph nodes draining that site, (2) measurement of cell proliferation in the draining lymph node, (3) absence of a skin reaction that could be indicative of the onset of the elicitation phase of skin sensitization, (4) data collected at the level of the individual animal to allow for an estimate of the variance within control and treatment groups (using this variance, a power analysis needs to be conducted to demonstrate that the modified method is utilizing a sufficient number of animals per treatment group to permit hazard identification with at least 95% power), and (5) if dose response information is needed, there are an adequate number of dose groups ($n \geq 3$) with which to accurately characterize the dose response for a given test substance.

The Panel noted that the list of substances included in the draft ICCVAM LLNA Performance Standards was sufficiently representative of the types of materials that are likely to be tested for skin sensitization. However, among the 13 sensitizers in the list of "required" substances, only five were considered to have robust data (i.e., traditional LLNA data based on at least three independent studies).

To evaluate performance for use in hazard identification, the Panel concluded that all 22 substances in the draft ICCVAM-recommended list should be tested and accuracy statistics calculated (Note: this list of substances includes "required" substances as well as "optional" false negative and false positive substances, of which only 8/22 have "robust" datasets [$n \geq 3$ as defined by the Panel]). To the extent possible, a rationale for any discordant results should be provided. However, the most potent sensitizers (e.g., dinitrochlorobenzene [DNCB]) should always be identifiable. Also, considerable weight should be given to the balance between animal welfare and human safety when considering the adequacy of test method accuracy. Based on the limited data available for the sensitizers on the list and the lack of standardization of test methods from which the results were obtained, the current database does not support inclusion of ECt values as a component of the accuracy evaluation.

The Panel agreed with the draft ICCVAM recommendations for evaluating test method reliability. These recommendations included obtaining ECt values that are generally within 0.5x to 2.0x of the mean historical EC3 (i.e., estimated concentrations needed to produce an SI of 3) values for hexyl cinnamic aldehyde (HCA) (intralaboratory, n=4 experiments in one laboratory), or HCA and DNCB (interlaboratory, n=1 experiment in three laboratories). However, the Panel recommended that the criteria for independent tests should be specified

(e.g., different animal shipment, different reagents, different operator). The Panel concluded that the proposed criteria for acceptability appeared to be appropriate in this case, because only one or two substances were being evaluated (i.e., a statistical multiple comparisons[2] problem does not exist). The Panel also suggested that historical control data using HCA and DNCB in the same vehicle could be used to demonstrate adequate intra- and/or inter-laboratory reproducibility.

The Panel also recommended that statistical tests to analyze the data might allow for a more accurate interpretation. They recommended that a suitable variance-stabilizing transformation (e.g., log transformation, square root transformation) be applied in all statistical analyses and in reporting summary standard deviations. The Panel also recommended that a more rigorous evaluation be conducted of what would be considered an appropriate range of ECt values to include as a requirement. This would be a statistical evaluation that considers the variability of ECt values generated among the sensitizers included on the performance standards reference substances list and the statistical multiple comparisons problem.

Use of the LLNA for Potency Determinations

The Panel agreed with the draft ICCVAM recommendation that the LLNA should not be used as a stand-alone assay for categorizing skin sensitizers as strong vs. weak, but that it could be used as part of a weight-of-evidence evaluation (e.g., along with quantitative structure-activity relationships, peptide reactivity, human evidence, historical data from other experimental animal studies) for this purpose. The Panel also agreed with the draft ICCVAM recommendation that any LLNA studies conducted for the purpose of evaluating skin sensitization potency should use the ICCVAM-recommended LLNA protocol. In addition, the Panel viewed that the relevant testing guidelines for the traditional LLNA should be revised to include the procedure for calculating an EC3 value.

A draft BRD was compiled by ICCVAM that provided a comprehensive review of available data and information and an evaluation of the usefulness and limitations of the traditional LLNA for the categorization of substances with regard to skin sensitization potency. The Panel evaluated the draft BRD for completeness, errors, and omissions and noted alternative analyses that would allow for a more complete evaluation of the use of the traditional LLNA for skin sensitization potency categorizations (see below).

The Panel agreed that the database of substances evaluated for potency determinations was sufficient and represented a range of chemical classes and physicochemical properties applicable to products typically tested for skin sensitization potential. The Panel also concluded that since the database was compiled from existing data, the lack of substance coding likely had no impact on the retrospective evaluation presented in the draft BRD. Still, the Panel recommended the coding of test substances in any future validation studies. The

[2] When multiple experiments are conducted and multiple observations, comparisons, or hypothesis tests are conducted, the chance of observing rare events increases. Suppose, for example, that an interval is established such that 5% of observations from a particular population of data are outside that interval. Then if k independent experiments generate data from this population (e.g., a standard normal distribution), the chances that all 20 results will lie inside the interval is $(1.0 - 0.05)^k$ (N. Flournoy, personal communication).

Panel generally agreed that potency determinations based on traditional LLNA results should ideally be limited to data from studies that evaluated lymph node proliferation in individual animals so that outliers and technical errors could be identified. However, they also agreed that pooled animal data should not be excluded automatically from a retrospective analysis.

The Panel indicated that the relevance of the LLNA for potency determinations had been adequately compared and evaluated to human (i.e., HMT or HRIPT) and guinea pig (i.e., GPMT or BT) data. A minority opinion stated by one Panel member was that the relevance of the traditional LLNA to human clinical observations had not been sufficiently determined.

In general, the Panel agreed that the proposed two-level categorization scheme (weak vs. strong sensitizers) for both human and guinea pig data was appropriate. However, a minority opinion stated by two Panel members was that a moderate category should be included since certain compounds might be on the border between weak and strong sensitizers. Thus, they suggested that the five-category scheme proposed by Kimber et al. (2003), which includes non-sensitizers, might be recommended.

The Panel concluded that the decision criteria providing the best overall performance was the use of <250 $\mu g/cm^2$ to distinguish between strong and weak sensitizers in humans and the use of an LLNA EC3 $\leq 9.4\%$ to distinguish between strong and weak sensitizers in the LLNA. The Panel stated that more data would be needed to determine if values different from these two would be more appropriate. The Panel also recommended that safety factors other than 10 for the lowest observed effect level (LOEL) be evaluated to determine if improved results could be obtained. The Panel also suggested an analysis that directly compares the LOEL values without using a safety factor (i.e., using LOEL data only) and an analysis that only uses no observed effect level data. The Panel further stated that traditional LLNA tests based on pooled or individual lymph nodes for a dose group should be evaluated independently to assess the impact of using pooled data on the accuracy analysis for skin sensitization potency. Finally, the Panel stated that the effect of different vehicles should be recognized as a limitation in the current data analysis and a likely contributor to the variability observed within and across laboratories.

The Panel stated that data from studies that could not be confirmed as being GLP-compliant, but that were from peer-reviewed literature or sources with high-quality laboratory management practices, were still appropriate to include in the accuracy analysis. However, the Panel stated that, ideally, GLP compliance should be the standard, as it is clearly the only objective way to judge the credibility of the data.

The Panel recommended that more data should be collected to determine the optimal threshold in humans for distinguishing between strong and weak sensitizers. In addition, the Panel discouraged conducting additional animal studies unless such studies would be expected to lead to an overall reduction in animal use. The Panel recommended that the LOELs from Akkan et al. (2003) be used instead of the DSA_{05} (i.e., the dose per skin area leading to a sensitization incidence of 5%) values from Schneider and Akkan (2004) in all of the potency analyses. A minority opinion by one Panel member stated that it was acceptable to use the DSA_{05} values from Akkan et al. (2003) as LOEL values in the evaluation. This panelist mentioned that the DSA_{05} value is a LOEL value adjusted to 5% incidence of induction in order to correct for human studies leading to different inductions. Furthermore, the panelist stated that because the DSA_{05} is corrected for an induction rate of 5%, it would

be better to compare with the traditional LLNA EC3 than to use the default uncorrected LOEL.

Appendix A

Peer Review Panel Member Biosketches

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Panel Member Biosketches

Nathalie Alépée, Ph.D.

Dr. Alépée performed research leading to a Ph.D. in Medical Virology and Microbiology at the Centre National de la Recherché Scientifique research institute, Gif sur Yvette, France. She is currently the Global Pfizer Leader for photosafety, including the global portfolio support and Associate Research Fellow in Investigative Toxicology, at Pfizer Global Research and Development, Amboise, France. As a laboratory manager in the Molecular and Cellular Toxicology Group with Pfizer, she implemented the Local Lymph Node Assay (LLNA) in the laboratory. She serves on the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee (ESAC), representing the European Federation of Pharmaceutical Industries Associations (EFPIA). She is also the Pfizer representative to the European Partnership on Alternative to Animal Testing (EPAA), in two working groups; Identification of Opportunities, Including R&D (working group 2), and Validation and Acceptance (working group 5). She served as a peer reviewer of the reduced LLNA test protocol and prediction model for ESAC in 2007 and has been designated as an ESAC peer reviewer for ECVAM's performance standards for the standard LLNA.

Anne Marie Api, Ph.D.

Dr. Api received a Ph.D. from Aston University in Birmingham, England and is currently Vice President of Human Health Sciences at the Research Institute for Fragrance Materials (RIFM), as well as the Scientific Director. She is responsible for the human health scientific program, and the investigation and initiation of new research and testing projects for RIFM. She is also Adjunct Assistant Professor at the University of Medicine and Dentistry of New Jersey. She is a member of 10 professional organizations, including the American Contact Dermatitis Society, the European Society of Contact Dermatitis, and the Society of Investigative Dermatology. She participated in the World Health Organization (WHO) International Workshop in Skin Sensitization in Chemical Risk Assessment held in Berlin, Germany in 2006. She is author of over 100 publications and presentations relevant to dermatology and dermatotoxicology.

Nancy Flournoy, M.S., Ph.D.

Dr. Flournoy received a M.S. degree in Biostatistics from the University of California at Los Angeles, and a Ph.D. in Biomathematics from the University of Washington. She is Professor and Chair of the Department of Statistics at the University of Missouri-Columbia. Her research interests include adaptive designs, bioinformatics, chemometrics, clinical trials, and environmetrics. She has an extensive list of edited volumes and papers on statistical theory, statistical genetics and immunology, epidemiology in immune suppressed subjects, clinical trials for prevention and treatment of viral infection, transplantation biology and its effects on digestion, lungs, eyes, mouth, and central nervous system, optimization of statistical processing, and additional papers, interviews, and technical reports. She has editorial responsibilities for numerous statistical journals, serves on numerous advisory boards, and nominating committees. She is a member and past Chair of the Council of Sections of the American Statistical Association, and served in various other statistical, medical and toxicological societies or programs as Chair or as a member of the Board of Directors. She is a former member of the Scientific Advisory Committee on Alternative Toxicological

Methods. She also served on the Expert Panels for the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) that evaluated the Revised Up-and-Down Procedure; the Current Validation Status of *In Vitro* Test Methods for Identifying Ocular Corrosives and Severe Irritants; and Five *In Vitro* Pyrogen Test Methods.

Thomas Gebel, Ph.D.

Dr. Gebel received a Ph.D. in Toxicology from the University of Mainz and is certified as a toxicologist by the German Society of Toxicology. His scientific interests are in biomonitoring, genetic toxicology, environmental hygiene, and occupational toxicology. He has published over 40 papers in peer-reviewed scientific journals. He is employed by the German Federal Institute for Occupational Safety and Health, and is an Associate Professor at the University of Goettingen. Dr. Gebel is currently a member of the Organisation for Economic Co-operation and Development (OECD) Globally Harmonized System of Classification and Labeling of Chemicals (GHS) expert group on sensitization and head of the German advisory committee on classification and labeling of existing substances and biocides. Dr. Gebel also is head of the German Delegations to the United Nations Economic and Social Council Sub-Committee of Experts on the GHS, and to the OECD Task Force on Harmonisation of Classification and Labeling. He participated in the WHO International Workshop in Skin Sensitization in Chemical Risk Assessment held in Berlin, Germany in 2006.

Sidney Green Ph.D., F.A.T.S.

Dr. Green received a Ph.D. in Biochemical Pharmacology from Howard University. His research interests include toxicology, mutagenic assay systems, and alternatives to animals in toxicology. He is currently Graduate Professor of Pharmacology at Howard University and a faculty member at the Centers for Alternatives to Animal Testing at the Johns Hopkins University School of Public Health. Previously, he has been Director of the Department of Toxicology at Covance Laboratories Inc. and the Director of the Division of Toxicological Research at the U.S. Food and Drug Administration (FDA). Dr. Green is a Fellow of the Academy of Toxicological Sciences (F.A.T.S.). He has served on numerous expert panels and committees. He was a participant in an International Workshop organized by ICCVAM and NICEATM on *In Vitro* Methods for Assessing Acute Systemic Toxicity in 2000. He served on the ICCVAM/NICEATM Expert Panels that evaluated the Corrositex® Test Method for Assessing Dermal Corrosivity Potential of Chemicals, and *In Vitro* Test Methods for Identifying Ocular Corrosives and Severe Irritants. He is a former member of the ICCVAM Advisory Committee on Alternative Toxicological Methods (ACATM) and of SACATM. He has authored over 60 publications for peer-reviewed journals.

Kim Headrick, B.Admin., B.Sc.

Kim Headrick received Bachelor of Administration and B.Sc. degrees from the University of Ottawa, Canada. She is currently International Harmonization and Senior Policy Advisor for Health Canada, and Chair of the UN Sub-Committee of Experts on GHS. She manages the overall strategy for the implementation of the GHS in Canada. She was awarded the Queen Elizabeth Commemorative Golden Jubilee Medal in 2002, which focuses on the achievements of people who, over the past 50 years, have created the Canada of today. She is

a member of the OECD Task Force on Harmonization of Classification and Labelling and the OECD Expert Group Meeting on Sensitization Hazards.

Dagmar Jírová, M.D., Ph.D.

Dr. Jírová received a Ph.D. from the Medical Faculty of Hygiene at Charles University in Prague. She is currently the Head of the Reference Center for Cosmetics, and Head of National Reference Laboratory for Experimental Immunotoxicology at the National Institute of Public Health in the Czech Republic. Her main responsibilities include safety assessment of consumer products, particularly cosmetics and their ingredients, performance of toxicological methods *in vivo* in animals, human patch testing for local toxicity assessment, and introduction of *in vitro* techniques for screening of toxicological endpoints using cell and tissue cultures. She represents the Czech Republic in the Standing Committee on Cosmetics of the European Commission. She is an ESAC-ECVAM member and was involved in Peer Review Panel for Skin Irritation Validation Study and LLNA test protocol and prediction model. She is author of more than 100 publications and presentations relevant to dermatotoxicology including a recent presentation at the 6th World Congress on Alternatives & Animal Use in the Life Sciences, held in Tokyo, 2007, titled "Comparison of Human Skin Irritation and Photoirritation Patch Test Data with Cellular *in vitro* Assays and Animal *in vivo* data".

David Lovell, Ph.D., B.Sc. (Hons), F.S.S., FIBiol, CStat, CBiol

Dr. Lovell received a Ph.D. from the Department of Human Genetics and Biometry, University College, London. He is currently Reader in Medical Statistics at the Postgraduate Medical School at the University of Surrey. Previously, he was Associate Director and Head of Biostatistics support to Clinical Pharmacogenomics at Pfizer Global Research and Development in Sandwich, Kent providing data management and statistical support to pharmacogenetics and genomics. He joined Pfizer in 1999 as the Biometrics Head of Clinical Pharmacogenetics. Before joining Pfizer, Dr. Lovell was the Head of the Science Division at BIBRA International, Carshalton, which included Molecular Biology, Genetic Toxicology, Biostatistics and Computer Services. At BIBRA, Dr. Lovell managed the statistical and computing group providing specialized statistical support to BIBRA's Clinical Unit and contract research work. He conducted and managed research programs on genetics, statistics and quantitative risk assessment for the European Union (EU) and U.K. Government Departments. His research interests at BIBRA were in the use of mathematical and statistical methods together with genetic models in the understanding of toxicological mechanisms and risk assessment problems. Dr. Lovell had previously been a Senior Research Officer with the U.K. Medical Research Council (MRC) Experimental Embryology and Teratology Unit, a visiting Postdoctoral Fellow at the National Institute of Environmental Health Sciences (NIEHS) in North Carolina, U.S., a Geneticist at the MRC Laboratories, Carshalton, and a Research Assistant in Cytogenetics at Birmingham University. He has acted as a consultant to a number of organizations, has considerable experience of working with Regulatory Authorities, has many publications related to his work and has wide experience of making presentations to a wide range of audiences. He is a member of the U.K. Government's advisory Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) and the Independent Scientific Advisory Committee for Medicines and Healthcare Products Regulatory Agency database research. He served on the NICEATM-ICCVAM Expert Panels

that evaluated the Frog Embryo Teratogenesis Assay - Xenopus, *In Vitro* Test Methods for Identifying Ocular Corrosives and Severe Irritants, and Five *In Vitro* Pyrogen Test Methods.

Michael Luster, Ph.D.

Dr. Luster received a Ph.D. in Immunology from Loyola University of Chicago. He was formerly Chief, Toxicology and Molecular Biology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health (NIOSH), and currently serves as a senior advisor to the Director of the Health Effects Laboratories and the staff of Toxicology and Molecular Biology Branch at NIOSH. Program areas include neuroscience, dermatology, molecular carcinogenesis, molecular epidemiology, molecular toxicology, molecular epidemiology, and inflammation/immunotoxicology. In addition, Dr. Luster conducts basic and applied research in immunotoxicology including its application in risk assessment. Current research activities include molecular epidemiology studies of genetic polymorphism involved in workplace-related diseases and experimental studies involving occupational allergic rhinitis. Dr. Luster is also working with various staff at the U.S. Environmental Protection Agency (EPA) through the Risk Assessment Forum to develop immunotoxicity testing guidelines. He also directed two studies for the NTP on the Toxicology and the Carcinogenesis of Promethazine and Ortho-phenylphenol, in 1990 and 1986, respectively. He is a co-author of over 300 publications in peer-reviewed journals.

Howard Maibach, M.D.

Dr. Maibach received an M.D. from Tulane University. He is currently a professor in the Department of Dermatology at the University of California, San Francisco (USCF), where he is also Chief of the Occupational Dermatology Clinic. In his 35 years at UCSF, Dr. Maibach has written and lectured extensively on dermatotoxicology and dermatopharmacology. His current research programs include defining the chemical-biologic faces of irritant dermatitis and the study of percutaneous penetration. Dr. Maibach served on the 1998 ICCVAM Peer Panel that evaluated the Murine LLNA. Dr. Maibach has been on the editorial boards of over 30 scientific journals and is a member of 19 professional societies including the American Academy of Dermatology, San Francisco Dermatological Society, and the International Commission on Occupational Health. He has co-authored over 1500 publications related to dermatology.

James McDougal, Ph.D., F.A.T.S.

Dr. McDougal earned a Ph.D. in Pharmacology/Toxicology at the University of Arizona. He is currently Professor and Director of Toxicology Research in the Department of Pharmacology and Toxicology at Wright State University's Boonshoft School of Medicine. Prior to his appointment at Wright State, he worked in the Air Force toxicology research organization for about 17 years. He has active skin research programs related to dermal pharmacokinetics, molecular biology of skin irritation, dermal risk assessment, and biologically-based mathematical modeling. He has served on many national committees, published more than 75 manuscripts, and consults for a wide variety of government and industry organizations. Dr. McDougal is a member of the National Academy of Sciences (National Research Council) Committee on Toxicology and the American Congress of Governmental Industrial Hygienists Threshold Limit Value Committee for Chemical substances. Dr. McDougal is also past president of the Dermal Toxicology Specialty Section of the Society of Toxicology.

Michael Olson, Ph.D., A.T.S.

Dr. Olson received a Ph.D. in Toxicology from the University of Arkansas for Medical Sciences, with dissertation research conducted at the FDA National Center for Toxicological Research. Following graduate training, he served as NIEHS National Research Service Award Post-doctoral Fellow in the Department of Pharmacology, School of Medicine - University of North Carolina. Currently he is Director, Occupational Toxicology, Corporate Environment Health and Safety for GlaxoSmithKline. Dr. Olson is a Fellow of the Academy of Toxicological Sciences (A.T.S.). His research interests include mechanisms of chemically-induced toxicity; genetic toxicity; xenobiotic metabolism; alternative methods in toxicology; hazard evaluation, risk assessment, and communication. Dr. Olson has authored a number of peer-reviewed manuscripts and book chapters in these areas as well as preparing many occupational health effects reviews for pharmaceutical active ingredients, isolated intermediates, and associated chemicals. He has served as an editorial board member and *ad hoc* referee for numerous toxicology and biosciences journals. In addition, he has worked as a Visiting Scientist, EPA, as well as advisor to EPA Risk Assessment Forum, U.S. National Institutes of Health (NIH) (Toxicology Study Section I), U.S. Air Force, Transportation Research Board, and the National Research Council - National Academy of Sciences. A member of several biomedical professional societies, Dr. Olson has served in elective and appointed positions in the Society of Toxicology, including Chairman of the Society of Toxicology (SOT) Occupational Health Specialty Section.

Raymond Pieters, Ph.D.

Dr. Pieters received a Ph.D. at Utrecht University and is currently an Associate Professor at the Institute for Risk Assessment Sciences, and Group Leader for Immunotoxicology at that institution. In 2007, he presented a paper on Development of Strategies to Assess Drug Hypersensitivity at the Congress of the European Societies of Toxicology. He was involved in the development of the Reporter Antigen Popliteal Lymph Node Assay, an assay to assess the immunomodulating potential of chemicals, which enables differentiation between immunosensitizing chemicals (sensitizers), immunostimulating chemicals (irritants), and chemicals that have no apparent immunological effects. He has published over 70 papers on sensitization and other subjects in immunotoxicology in peer-reviewed journals, including a review article, *Murine Models of Drug Hypersensitivity*, in 2005.

Jean Regal, Ph.D.

Dr. Regal received a Ph.D. in Pharmacology from the University of Minnesota. She is currently a Professor in the Department of Pharmacology, Department of Biochemistry & Molecular Biology and Associate Dean of Faculty Affairs, Medical School Duluth, University of Minnesota. Her current research is focused on respiratory allergy, especially asthma. She has served on multiple NIH review panels regarding asthma, as an immunotoxicologist in 2000 for an Institute of Medicine Committee on Health Effects Associated with Exposures Experienced during the Persian Gulf War, as well as on the 1998 ICCVAM Peer Panel that evaluated the Murine LLNA. In 2007 she served as an ad hoc reviewer for the NTP Board of Scientific Counselors for two nominations: Artificial Butter Flavoring Mixture & O-phthalaldehyde, at NIEHS. Also in 2007, she served on an NIEHS Center in Environmental Toxicology pilot project program for the University of Texas Medical Branch at Galveston. She is currently Vice-President-elect of the Immunotoxicology

Specialty Section of SOT and Associate Editor of the Journal of Immunotoxicology. Dr. Regal has authored over 50 research articles and reviews in peer-reviewed journals and holds two patents on pulmonary administration of sCR1 and other complement inhibitory proteins.

Jonathan Richmond, B.Sc. (Hons) Med.Sci., MB ChB, FRCSEd, FRMS

Dr. Richmond received a Bachelor of Science in Medical Science with Honors (B.Sc. [Hons] Med.Sci.) and Bachelor of Medicine and Bachelor of Surgery (MB ChB) degrees with Distinction in Medicine and Therapeutics from Edinburgh University. Presently, he is head of the Animals Scientific Procedures Division at the Home Office. He is a Fellow of the Royal College of Surgeons of Edinburgh (FRCSEd) and a Fellow of the Royal Society of Medicine (FRMS). Other appointments include convener of the U.K. interdepartmental group on the 3Rs, board member U.K. National Centre for the 3Rs, convener of the International Standards Organization Technical Corrigendum 194/Working Group 3 (*Biocompatibility of Medical Device Materials*), and member of related expert working groups. He is a former member of the EU Committee on Scientific and Technical Progress and past Chairman of the European Commission Technical Expert Working Group on ethical review. He served as chair of the peer review panel for the reduced LLNA test protocol and prediction model for ESAC in 2007 and has been designated as an ESAC peer reviewer for ECVAM's performance standards for the standard LLNA. He served on the ICCVAM/NICEATM Expert Panel that evaluated Five *In Vitro* Pyrogen Test Methods. He has a variety of publications in peer-reviewed journals and national and international meetings, on the principles and practice of surgery, regulation of biomedical research, principles of humane research, bioethics, and public policy.

Peter Theran, V.M.D.

Dr. Theran holds a Doctor of Veterinary Medicine degree from the University of Pennsylvania. He has had many years of experience both as a veterinary internal medicine specialist at the Massachusetts Society for Prevention of Cruelty to Animals' Angell Memorial Animal Hospital in Boston, and as the director of Boston University Medical Center's Laboratory Animal Science Center. He presently serves on a number of government committees as an animal welfare member, and is a member of the Board of Directors of the Institute for *In Vitro* Sciences in Gaithersburg, MD and Chimp Haven in Shreveport, Louisiana. He served on the NICEATM-ICCVAM Expert Panels that evaluated the *In Vitro* Test Methods for Identifying Ocular Corrosives and Severe Irritants, and Five *In Vitro* Pyrogen Test Methods. He is a former member of ACATM and SACATM. He is presently working as a consultant.

Stephen Ullrich, Ph.D.

Dr. Ullrich received a Ph.D. in Microbiology from Georgetown University. He is currently the Dallas/Fort Worth Living Legends Professor, and Professor of Immunology at the University of Texas, M.D. Anderson Cancer Center, where he is also Associate Director, The Center for Cancer Immunology Research. He is also a member of the Animal Research Strategic Advisory Committee. He has served numerous national review committees and panels, including the 1998 ICCVAM Peer Panel that evaluated the Murine LLNA. Dr. Ullrich has authored over 75 peer-reviewed publications, over 30 invited articles, and he holds four patents in the U.S., E.U., and Australia for a UV-induced Immunosuppressive Substance. He is the past President of the American Society for Photobiology.

Michael Woolhiser, Ph.D.

Dr. Woolhiser received a Ph.D. in Pharmacology and Toxicology from the Medical College of Virginia at Virginia Commonwealth University. He is a specialist in immunotoxicology and is currently a toxicologist for the Dow Chemical Company where he serves as a Technical Leader for Immunotoxicology, and Polyurethane Business Toxicology Consultant. Dr. Woolhiser is also an Adjunct Professor at the Center for Integrative Toxicology, Michigan State University. He is a member of the Program Committee of the Society of Toxicology's Immunotoxicology Specialty Section. He has served on numerous working groups, including an LLNA Expert Working Group under the European Crop Protection Agency's Toxicology Expert Group, a European Centre for Ecotoxicology and Toxicology of Chemicals LLNA Task Force. He has authored 29 peer-reviewed publications.

Takahiko Yoshida, M.D., Ph.D.

Dr. Yoshida earned his M.D. and a Ph.D. in Medical Science from Tokai University. He is currently Professor in the Department of Health Science at Asahikawa Medical College. Prior to this appointment, he held the posts of Instructor, Assistant Professor and Associate Professor at the Tokai University School of Medicine. He has also been a Guest Researcher at NIEHS. He has also worked as an occupational physician for major Japanese corporations, including Toyota and Sony. Dr. Yoshida’s research interests include occupational health, public health, environmental health and preventative medicine. He is a member of the International Congress of Occupational Health, the Japanese Society of Hygiene, the Japanese Society of Immunotoxicology, the Japanese Society of Clinical Ecology, and the SOT.

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Appendix D3

Summary Minutes of Independent Scientific Peer Review Panel Meeting on April 28-29, 2009

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Summary Minutes

Independent Scientific Peer Review Panel Meeting

Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Evaluation of the Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA)

William H. Natcher Conference Center
National Institutes of Health
Bethesda, MD
April 28 - 29, 2009
8:30 a.m. - 5:30 p.m.

Peer Review Panel Members:

Michael Luster, Ph.D. (Peer Review Panel Chair)	Senior Consultant to the NIOSH Health Effects Laboratory, Morgantown, WV
Nathalie Alépée, Ph.D.	Scientific Coordinator on Alternatives Methods in Life Science, L'Oréal Research and Development, Aulnay sous Bois, France
Anne Marie Api, Ph.D.	Vice President, Human Health Sciences, Research Institute for Fragrance Materials, Woodcliff Lake, NJ
Nancy Flournoy, M.S., Ph.D.	Professor and Chair, Dept. of Mathematics and Statistics, University of Missouri – Columbia, Columbia, MO
Dagmar Jírová, M.D., Ph.D.	Toxicologist, Research Manager, Head of Reference Center for Cosmetics, Head of Reference Laboratory for Experimental Immunotoxicology, National Institute of Public Health, Czech Republic
David Lovell, Ph.D.	Reader in Medical Statistics, Postgraduate Medical School, University of Surrey, Guildford, Surrey, U.K.
Howard Maibach, M.D.	Professor, Dept. of Dermatology, University of California – San Francisco, San Francisco, CA
Michael Olson, Ph.D.	Director of Occupational Toxicology, Corporate Environment Health and Safety, GlaxoSmithKline, Research Triangle Park, NC

Peer Review Panel Members:

Raymond Pieters, Ph.D.[1]	Associate Professor, Immunotoxicology Group Leader, Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands
Jean Regal, Ph.D.	Professor, Dept. of Pharmacology, University of Minnesota Medical School, Duluth, MN
John Richmond, MB ChB, FRCSEd	Head, Animals Scientific Procedures Division, Home Office, London, U.K.
Peter Theran, V.M.D.	Massachusetts Society for the Prevention of Cruelty to Animals, Novato, CA
Stephen Ullrich, Ph.D.	Dallas/Ft. Worth Living Legends Professor and Professor of Immunology, Postgraduate School of Biomedical Science, University of Texas M.D. Anderson Cancer Center, Houston, TX
Michael Woolhiser, Ph.D.	Science and Technology Leader – Toxicology and Environmental Research and Consulting, The Dow Chemical Company, Midland, MI
Takahiko Yoshida, M.D., Ph.D.	Professor, Dept. of Health Science, Asahikawa Medical College, Hokkaido, Japan

ICCVAM and ICCVAM Immunotoxicity Working Group Members:

Paul Brown, Ph.D.	FDA, Center for Drug Evaluation and Research, Silver Spring, MD
Masih Hashim, Ph.D.	EPA, Office of Pesticide Programs, Washington, DC
Ying Huang, Ph.D.	FDA, Center for Biologics Evaluation and Research, Silver Spring, MD
Abigail Jacobs, Ph.D. (IWG Co-Chair)	FDA, Center for Drug Evaluation and Research, Silver Spring, MD
Jodie Kulpa-Eddy, D.V.M.	USDA, Animal and Plant Health Inspection Service, Riverdale, MD
Elizabeth Margosches, Ph.D.	EPA, Office of Pollution Prevention and Toxics, Washington, DC
Joanna Matheson, Ph.D. (IWG Co-Chair)	CPSC, Bethesda, MD

[1] Dr. Pieters was unable to attend the public meeting on April 28-29, 2009. However, he was involved in the review of the revised draft background review documents and the revised draft LLNA applicability domain Addendum.

ICCVAM and ICCVAM Immunotoxicity Working Group Members:

Deborah McCall	EPA, Office of Pesticide Programs, Washington, DC
Tim McMahon, Ph.D.	EPA, Office of Pesticide Programs, Washington, DC
John Redden, M.S.	EPA, Office of Pesticide Programs, Washington, DC
R. Adm. William Stokes, D.V.M., DACLAM	NIEHS, Research Triangle Park, NC
Ron Ward, Ph.D.	EPA, Office of Pollution Prevention and Toxics, Washington, DC
Marilyn Wind, Ph.D. (ICCVAM Chair)	CPSC, Bethesda, MD

Invited Experts:

George DeGeorge, Ph.D., DABT	MB Research Labs, Spinnerstown, PA
Kenji Idehara, Ph.D.	Daicel Chemical Industries, Ltd., Hyogo, Japan
Masahiro Takeyoshi, Ph.D.	Chemicals Evaluation and Research Institute, Saitama, Japan

JaCVAM Observer:

Hajime Kojima, Ph.D.	National Institute of Health Sciences, Tokyo, Japan

Public Attendees:

Joan Chapdelaine, Ph.D.	Calvert Laboratories, Inc., Olyphant, PA
Merrill Tisdel	Syngenta Crop Protection Inc., Greensboro, NC
Gary Wnorowski, M.B.A, L.A.T.	Eurofins Product Safety Labs

NICEATM:

R. Adm. William Stokes, D.V.M., DACLAM	Director
Debbie McCarley	Special Assistant to the Director

Contract Support Staff – Integrated Laboratory Systems, Inc. (ILS)

David Allen, Ph.D.	Eleni Salicru, Ph.D.
Thomas Burns, M.S.	Frank Stack

NICEATM:

Linda Litchfield

Judy Strickland, Ph.D., DABT

Greg Moyer, M.B.A.

Abbreviations:

CPSC = U.S. Consumer Product Safety Commission

EPA = U.S. Environmental Protection Agency

FDA = U.S. Food and Drug Administration

ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods

ILS = Integrated Laboratory Systems

IWG = Immunotoxicity Working Group

NICEATM = National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

NIEHS = National Institute of Environmental Health Sciences

NIOSH = National Institute of Occupational Safety and Health

USDA = U.S. Department of Agriculture

Tuesday, April 28, 2009
Call to Order and Introductions

Dr. Michael Luster (Peer Review Panel Chair) called the meeting to order at 8:30 a.m. and introduced himself. He then asked all Peer Review Panel (hereafter Panel) members to introduce themselves and to state their name and affiliation for the record. He then asked all the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) staff, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) members, the ICCVAM Immunotoxicity Working Group (IWG) members, and members of the public to also introduce themselves. Dr. Luster stated that there would be opportunity for public comments during each of the four murine local lymph node assay (LLNA)-related topics. He asked that all those interested in making a comment register at the registration table and provide a written copy of their comments, if available, to NICEATM staff. Dr. Luster emphasized that the comments would be limited to seven minutes per individual and that, while comments from one individual would be welcomed during each commenting period, repeating the same comments at each comment period would be inappropriate.

Welcome from the ICCVAM Chair

Dr. Marilyn Wind, U.S. Consumer Product Safety Commission (CPSC) and Chair of ICCVAM, welcomed everyone to the National Institutes of Health and to the Panel meeting. Dr. Wind thanked the ICCVAM IWG and NICEATM staff for their efforts in preparing the draft documents being reviewed and for arranging the logistics of the meeting. Dr. Wind thanked the Panel members for dedicating their time, effort, and expertise to this review and acknowledged their important role to the ICCVAM test method evaluation process. Dr. Wind also emphasized the important role of the public and their comments in this process.

Welcome from the Director of NICEATM, and Conflict of Interest Statements

Dr. William Stokes, Director of NICEATM, stated the Panel meeting was being convened as an NIH Special Emphasis Panel and was being held in accordance with applicable U.S. Federal Advisory Committee Act regulations. As such, Dr. Stokes indicated that he would be serving as the Designated Federal Official for this public meeting. He reminded the Panel that they signed a conflict of interest (COI) statement during the Panel selection process, in which they identified any potential real or perceived COI. He read the COI statement and then Dr. Luster asked that panelists again declare any potential direct or indirect COI and to recuse themselves from discussion and voting on any aspect of the meeting where there might be a conflict.

Dr. Michael Woolhiser declared a COI regarding the Panel's review of the LLNA Applicability Domain, because The Dow Chemical Company, Dr. Woolhiser's employer, submitted much of the data that were being considered. He indicated that he would recuse himself from the Panel's evaluation of the applicability domain, but would remain available to answer any questions that the Panel might have about the test substances or the data.

Overview of the ICCVAM Test Method Evaluation Process

Dr. Stokes began by thanking the 15 Panel scientists from six different countries (Czech Republic, France, Japan, The Netherlands, United Kingdom, and the United States) for their significant commitment of time and effort preparing for and attending the meeting. He explained that the purpose of the Panel was to conduct an independent scientific peer review of the information provided on a series of proposed new versions of the LLNA and proposed expanded applications of the assay. The Panel is then asked to comment on the extent that the available information supports the draft ICCVAM recommendations. Dr. Stokes indicated that the original LLNA peer review panel in 1998 considered the LLNA a valid substitute for the guinea pig-based test in most but not all testing

situations. He noted that three Panel members from the 1998 review are also on the current Panel (i.e., Drs. Howard Maibach, Jean Regal, and Stephen Ullrich). Dr. Stokes also reviewed the nomination that was received from CPSC in January 2007,[2] which provides the basis for the current evaluation.

Dr. Stokes then identified the 15 Federal agencies that comprise ICCVAM and summarized ICCVAM's mission. He noted that ICCVAM, as an interagency committee, does not carry out research and development or validation studies. Instead, ICCVAM, in conjunction with NICEATM, carries out the critical scientific evaluation of the results of validation studies for proposed test methods to assess their usefulness and limitations for regulatory testing, and then makes formal recommendations to ICCVAM agencies.

Dr. Stokes provided a brief review of ICCVAM's history and summarized the ICCVAM Authorization Act of 2000,[3] including the purpose and duties of ICCVAM. He noted that one of ICCVAM's primary duties is to review and evaluate new, revised, and alternative test methods applicable to regulatory testing. He stated that all of the reports produced by NICEATM are available on the NICEATM-ICCVAM website or can be obtained upon request from NICEATM. He also mentioned that ICCVAM provides guidance on test method development, validation processes, and helps to facilitate not only the acceptance of scientifically valid alternative test methods, but also encourages internationally harmonized recommendations on the usefulness and limitations of alternative test methods.

Dr. Stokes then described the ICCVAM test method evaluation process, which begins with a test method nomination or submission. NICEATM conducts a prescreen evaluation to summarize the extent to which the proposed submission or nomination addresses the ICCVAM prioritization criteria. A report of this evaluation is then provided to ICCVAM, which in turn develops recommendations regarding the priority for evaluation. ICCVAM then seeks input on their recommendations from the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) and the public and determines whether the test method should move forward into a formal evaluation. If so, a draft background review document (BRD), which provides a comprehensive review of all available data and information, is prepared by NICEATM in conjunction with an ICCVAM working group designated for the relevant toxicity testing area (e.g., the IWG). In addition, ICCVAM considers all available information and develops draft test method recommendations on the proposed usefulness and limitations of the test methods, test method protocol, performance standards, and future optimization/validation studies. The draft BRD and the draft ICCVAM test method recommendations are made available to the Panel and the public for review and comment. The Panel peer reviews the draft BRD and evaluates the extent to which it supports the draft ICCVAM test method recommendations. A Panel report is published, which is then considered along with public and SACATM comments by ICCVAM in developing final recommendations. These final recommendations are forwarded to the ICCVAM member agencies for their consideration and possible incorporation into relevant testing guidelines. Agencies have 180 days to respond to the ICCVAM recommendations.

Dr. Stokes reviewed the ICCVAM criteria for adequate validation. He stated that validation is defined by ICCVAM as the process by which the reliability and relevance of a procedure are established for a specific purpose, and that adequate validation is a prerequisite for consideration of a test method by U.S. Federal regulatory agencies. Dr. Stokes listed the ICCVAM acceptance criteria for test method validation and acceptance. He concluded by summarizing the timeline of the review activities beginning with CPSC's nomination in January 2007 and ending with the present Panel meeting.

[2] http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf
[3] http://iccvam.niehs.nih.gov/docs/about_docs/PL106545.pdf

ICCVAM Charges to the Panel

Dr. Stokes reviewed the charges to the Panel: (1) review the draft BRDs and the draft Addendum to the traditional[4] LLNA for completeness and identify any errors or omissions; (2) determine the extent to which each of the applicable criteria for validation and regulatory acceptance had been appropriately addressed for the proposed revised or modified versions of the LLNA; and (3) comment on the extent to which the ICCVAM draft test method recommendations including the proposed usefulness and limitations, standardized test method protocols, performance standards, and additional studies are supported by the information provided in the draft BRDs and draft Addendum.

Overview of the Agenda

Dr. Luster then reviewed the agenda and the order of presentations. He stated that for each review topic, the test method developer would present an overview of the test method protocol, followed by a presentation by NICEATM staff summarizing each revised draft BRD, and lastly a member of the IWG would present the draft ICCVAM recommendations. Following presentations, the Panel Evaluation Group Leader for the topic under consideration would present the group's draft recommendations, followed by Panel discussion. Public comments would then be presented, followed by the opportunity for additional Panel discussion in consideration of the public comments. The Panel would then vote to accept the Panel consensus, with any minority opinions being so noted with the rationale provided for the minority opinion.

Current Regulatory Testing Requirements and Hazard Classification Schemes for Allergic Contact Dermatitis (ACD) and the Traditional LLNA Procedure

Dr. Matheson presented an overview of ACD and relevant regulatory requirements. She briefly discussed the ICCVAM final recommendations for the LLNA Performance Standards, the updated ICCVAM LLNA test method protocol, and the reduced LLNA (rLLNA), all of which were reviewed by the Panel at their meeting in March 2008.

The Panel questioned who was responsible for conducting the future studies referred to in the revised draft ICCVAM test method recommendations. Dr. Stokes replied that these recommendations are provided for consideration by the stakeholder community. Those organizations with appropriate resources can use this information to guide their research, development, and validation activities.

A question arose from the Panel as to why pooled data (as opposed to individual animal data) are collected for the LLNA.

Dr. Matheson replied that, pooled data are often collected since OECD Test Guideline 429 allows the use of a minimum of four animals per treatment group when collecting pooled data, but requires a minimum of five animals per treatment group when collecting individual animal data. Legislation in some countries, and many Animal Care and Use Committees, require that the test method to be used is the one requiring the fewest animals. Dr. Matheson also noted that the ICCVAM LLNA test method protocol has recently been revised to allow the use of a minimum of four animals per treatment group when collecting individual animal data, so there is now no reason not to collect individual animal data. At the Panel meeting in March 2008, the Panel stated that all future LLNA studies should require that lymph nodes be collected from individual animals instead of pooling them

[4] For the purposes of this document, the radioactive LLNA test method, which was first evaluated by ICCVAM in 1999, and subsequently recommended to U.S. Federal agencies as a valid substitute for currently accepted guinea pig test methods to assess the allergic contact dermatitis potential of many, but not all, types of substances, is referred to as the traditional LLNA.

with other animals in a treatment group since individual animal response data allows for identification of technical problems and outlier animals within a dose group.[5]

A question arose as to whether the U.S. Environmental Protection Agency (EPA) prefers LLNA or guinea pig data for submission. Dr. Matheson ceded the floor to Ms. Debbie McCall of EPA Office of Pesticide Programs, who was in attendance. Ms. McCall said that EPA prefers LLNA data, but will accept either guinea pig maximization test (GPMT) or Buehler test (BT) data.

Overview of the Revised Draft LLNA: DA Test Method Procedure BRD and Revised Draft ICCVAM Test Method Recommendations

The first test method reviewed was the LLNA: DA test method. This test method measures the ATP content of lymph node cells by the luciferin/luciferase method, as an index of lymphocyte proliferation, after exposure to a test substance.

Dr. Kenji Idehara of Daicel Chemical Industries, Ltd., Japan (the test method developer) presented a synopsis of the test method to the Panel.

A Panelist asked about the half-life of ATP in the lymph node cells after the mouse is sacrificed. Dr. Idehara replied that the ATP concentration declines 20 to 30% in an hour, with a half-life of about 2 to 2.5 hours. The assay time from animal sacrifice to complete measurement of ATP content for each individual animal is maintained as similar as possible, within approximately 30 min. He also said that the time between sacrifice and ATP assay is not a problem when collecting individual animal data, if the time between the excision of the lymph nodes, the preparation of the cell suspensions, and the measurement of the ATP concentrations is kept relatively constant between animals.

A Panelist asked if the lymph node samples were randomized before the ATP assays were conducted. Dr. Idehara replied that the samples were not randomized.

On behalf of NICEATM, Dr. Salicru presented an overview of the revised draft LLNA: DA BRD to the Panel.

A question arose about NICEATM's use of different decision criteria for the accuracy analysis, and the reproducibility analyses in the revised draft BRD. Dr. Salicru noted that a decision criterion of SI ≥ 2.5 was used for the reproducibility analyses because it was found to be the optimal decision criterion for identifying sensitizers (i.e., it resulted in a 0% false positive rate).

Dr. Wind presented the revised draft ICCVAM test method recommendations for the LLNA: DA test method to the Panel. She noted that ICCVAM favored the multiple decision criteria to eliminate any false positives or false negatives. A Panelist commented that, as more data are accumulated using the test method, false positives and false negatives might appear.

A Panelist asked, if the true stimulation index (SI) value for a compound was 2.0, if that compound would be classified as a sensitizer or a nonsensitizer. Dr. Wind replied that, as described in the revised draft ICCVAM recommendations, other information would be necessary to definitively answer that question.

Dr. Kojima presented the results of the Japanese Society for Alternatives to Animal Experiments (JSAAE) interlaboratory validation studies of the LLNA: DA and the LLNA: BrdU-ELISA test methods to the Panel. In the presentation, he noted that the JaCVAM Regulatory Acceptance Board has examined the results of the studies for both test methods and accepted the LLNA: DA as a replacement for the traditional LLNA. The JaCVAM Regulatory Acceptance Board has requested additional data for the LLNA: BrdU-ELISA.

[5] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

Panel Evaluation:

Dr. Woolhiser presented the draft position developed by Evaluation Group B, which was charged with primary review of the LLNA: DA test method. The Panel agreed that the available data and test method performance support the use of the LLNA: DA to identify substances as potential skin sensitizers and nonsensitizers, with certain limitations. They concurred with ICCVAM's proposal that, based on the current validation database, the multiple SI decision criteria should be used to identify sensitizers and nonsensitizers (i.e., SI ≥ 2.5 for sensitizers, SI ≤ 1.7 for nonsensitizers). The Panel also noted that the limitation of these test methods when using the proposed multiple decision criteria is the indeterminate classification of substances that fall in the range of SI values for which a classification is uncertain (i.e., 1.7 < SI < 2.5). The Panel recommended that when such results are obtained, users should carefully interpret the results using an integrated decision strategy in conjunction with all other available information (e.g., dose response and quantitative structure-activity relationship [QSAR] information, peptide-binding activity, molecular weight, results from related chemicals, other testing data) to determine if there is adequate information for an accurate sensitization hazard classification or if additional testing is necessary. The Panel emphasized that, from an animal welfare perspective, retesting should not be undertaken until all other available information is evaluated, and a determination is made that such testing is required to fill a data gap. The Panel also recommended that more detailed guidance be developed for regulatory agencies on how the multiple decision criteria could be used in practice.

Subsequent Panel discussions focused on ICCVAM's recommendation to use multiple decision criteria to identify sensitizers and nonsensitizers. In general, the Panel preferred the multiple decision criteria to a single decision criterion for identifying sensitizers and nonsensitizers. A Panelist recommended that graphs showing the maximum SI obtained with the modified test method (the LLNA: DA, in this case) plotted against the maximum SI obtained with the traditional LLNA, for each test substance, be included in the final BRD. This was a general recommendation for both test methods that use multiple decision criteria (i.e., the LLNA: DA and LLNA: BrdU-ELISA). It was also pointed out that, as more data are accumulated for these test methods, the cut-off SI values for sensitizers and nonsensitizers would likely change.

Bootstrapping analysis was mentioned as a means to provide some measure of variability of the chosen cut-off values. It was also mentioned that the tables in Section 7.0 of the revised draft BRD provide no measurement of variation for the data. It was suggested that all of these tables include treatment means, standard deviations, and the mean squares, so that F-values can be calculated for between and among laboratory means. However, the Panel agreed that, while this information would be useful for inclusion in the final BRD, it would not impact the Panel's overall conclusions about the test method.

Some discussion followed about variations in the LLNA: DA test method protocol from the updated ICCVAM-recommended traditional LLNA test method protocol (i.e., sodium lauryl sulfate pretreatment prior to test substance application and an additional test substance application on day 7). The Panel agreed that despite these variations, the LLNA: DA was still mechanistically and functionally similar to the traditional LLNA.

Public Comments:

At the conclusion of the Panel discussion, Dr. Luster called for public comments. None were presented.

Panel Conclusions and Recommendations:

Dr. Luster asked if the Panel was in agreement with the conclusions in the draft Panel Report as reflected in the updated Evaluation Group presentation as modified during the discussions. The Panel approved unanimously.

Applicability Domain of the LLNA and Revised Draft ICCVAM Test Method Recommendations

NICEATM provided an overview of the revised draft Addendum on the LLNA applicability domain. Subsequent to the 2008 Panel consideration of this topic, new data were obtained for pesticide formulations, dyes, essential oils, and substances tested in aqueous solution, but none were obtained for metals. Since the Panel previously considered the use of the term *mixtures* too broad, data were separately evaluated by product subgroups in the revised draft Addendum, and they were identified in general terms as pesticide formulations and other products. Dr. Wind presented the revised draft ICCVAM test method recommendations for the LLNA applicability domain to the Panel.

Subsequent to Dr. Wind's presentation, Dr. Luster asked Ms. McCall of EPA to clarify EPA's position on the use of LLNA data for pesticide formulations. Ms. McCall replied that EPA accepted positive or negative LLNA data on single substance technical grade additives. Between 2003 and 2007, EPA received few LLNA studies on pesticide formulations. Positive LLNA results were accepted, but for negative results, EPA required a confirmatory test. The majority of sensitization data submitted to EPA for pesticide formulations are from the guinea pig BT. There are limited human data available on pesticides due to the ethics limitations for conducting human studies, and applicants provide all of EPA's data.

A Panelist commented that the GPMT is more sensitive that the BT; he said that, in his experience, the GPMT showed roughly 60% positive results versus 20% positive results for the BT, for the same group of formulations. He said that the LLNA is more concordant with the GPMT than it is with the BT. He said that the GPMT is the preferred test in Europe. The Panel agreed that this should be reflected in the comparisons of LLNA and guinea pig results.

Panel Evaluation:

Dr. Olson presented the draft position developed by Evaluation Group A, which was charged with primary review of the LLNA applicability domain, to the Panel. While the Panel agreed that there were too few data in the revised draft Addendum for some of the test substance classes (e.g., dyes, essential oils) to make a firm statement about concordance of the LLNA with other test methods for these classes, the Panel stated that any material should be suitable for testing in the LLNA unless there is a biologically-based rationale for exclusion, such as unique physicochemical properties that might affect their ability to interact with immune processes. The Panel therefore agreed that the LLNA should be considered appropriate for testing pesticide formulations and other products, unless there is a biologically-based rationale for exclusion.

The Panel also concurred that, while studies done with BALB/c mice should not be excluded from the evaluations in the revised draft Addendum, CBA should remain the preferred strain for the updated ICCVAM-recommended LLNA test method protocol, and that the use of any other strain, or of male rather than female mice, should be justified by the investigator.

The Panel did not agree that Pluronic L92 should be added to the list of preferred vehicles for the LLNA, but it did agree that studies done with Pluronic L92 should not be excluded from the evaluations in the revised draft Addendum.

While the concordance of LLNA results for essential oils was properly compared with human results, the Panel noted that the revised draft Addendum neglected to consider information that showed LLNA results were more concordant with human results when the major component was ≥70%, compared to the concordance for the essential oil itself. The Panel also commented that the term *natural complex substances* was more appropriate for these types of substances than *essential oils*, because this is the terminology used for the Registration, Evaluation, Authorisation and Restriction of Chemical substances program now in force in the European Union (EU).

In reference to the data for the medical device eluates in the revised draft Addendum, the Panel commented that ISO Standard 1099 requires the chemical analysis of such materials before skin sensitization testing is undertaken, and therefore agreed that the data provided were of little use for evaluating the performance of the LLNA for testing these types of substances.

Public Comments:

At the conclusion of the Panel discussion, Dr. Luster called for public comments.

Mr. Gary Wnorowski, Eurofins Product Safety Labs
Mr. Gary Wnorowski said he had registered to make a public comment, but that Ms. McCall of EPA had already addressed his question by her answer to Dr. Luster's question regarding acceptability of pesticide formulation data.

Panel Conclusions and Recommendations:

Dr. Luster asked if the Panel was in agreement with the conclusions in the draft Panel Report as reflected in the updated presentation. The Panel approved unanimously.

Adjournment

At the conclusion of the discussion on the applicability domain, Dr. Luster adjourned the Panel for the day at 5:30 p.m., to reconvene at 8:30 a.m. on Wednesday, April 29, 2009.

Wednesday, April 29, 2009
Overview of the Draft LLNA: BrdU-ELISA Test Method Revised Draft BRD and Revised Draft ICCVAM Test Method Recommendations

Dr. Luster called for Panel consideration of the LLNA: BrdU-ELISA test method. This test method measures bromodeoxyuridine (BrdU), a thymidine analog, instead of radioactive thymidine, incorporated into the DNA of proliferating lymphocytes, via an enzyme-linked immunosorbent assay (ELISA).

Dr. Masahiro Takeyoshi of Chemicals Evaluation and Research Institute, Japan (the test method developer) presented a synopsis of the test method to the Panel.

On behalf of NICEATM, Dr. Strickland presented an overview of the revised draft ICCVAM LLNA: BrdU-ELISA BRD to the Panel.

A Panelist asked why ICCVAM proposes an SI value of 2.0 as the cutoff value for a sensitizer instead of a value of 2.5, since the data indicated that no false positives would result if either value were used. Dr. Strickland replied that the value of 2.0 was chosen because this was the lowest value that resulted in a 0% false positive rate, thus minimizing the range of uncertainty.

Dr. Jacobs presented the revised draft ICCVAM test method recommendations for the LLNA: BrdU-ELISA test method to the Panel.

Panel Evaluation:

Dr. Ullrich presented the draft position developed by Evaluation Group B, which was charged with primary review of the LLNA: BrdU-ELISA test method, to the Panel.

The Panel agreed that the LLNA: BrdU-ELISA test method was mechanistically and functionally similar to the traditional LLNA, and the ICCVAM LLNA Performance Standards could be used to evaluate it. The Panel also concurred that the available data and test method performance support the use of the LLNA: BrdU-ELISA to identify substances as potential skin sensitizers and nonsensitizers, with certain limitations. They agreed with ICCVAM's proposal that, based on the current validation database, the multiple SI decision criteria should be used to identify sensitizers and nonsensitizers

(i.e., SI ≥ 2.0 for sensitizers, SI > 1.3 for nonsensitizers). The Panel also noted that the limitation of these test methods when using the proposed multiple decision criteria is the indeterminate classification of substances that fall in the range of SI values for which a classification is uncertain (i.e., 2.0 > SI ≥ 1.3). The Panel recommended that when such results are obtained, users should carefully interpret the results in an integrated decision strategy in conjunction with all other available information (e.g., dose-response and QSAR information, peptide-binding activity, molecular weight, results from related chemicals, other testing data) to determine if there is adequate information for an accurate sensitization hazard classification or if additional testing is necessary. The Panel emphasized that, from an animal welfare perspective, retesting should not be undertaken until all other available information is evaluated, and a determination is made that such testing is required to fill a data gap. The Panel also recommended that more detailed guidance be developed for regulatory agencies on how the multiple decision criteria could be used in practice.

Subsequent Panel discussions focused on ICCVAM's recommendation to use multiple decision criteria to identify sensitizers and nonsensitizers. In general, the Panel preferred the multiple decision criteria to a single decision criterion for identifying sensitizers and nonsensitizers. The Panel agreed that all of the comments for the LLNA: DA test method regarding the graphs and tables in the revised draft BRD, and the provision of measures of variation for interlaboratory reproducibility data, apply to the BrdU-ELISA also.

A Panelist commented that the use of interpolation for determining ECt values presupposed a monotonic increase in SI values and that isotonic regression might be more appropriate in cases in which a monotonic increase does not occur. More Panel discussion occurred regarding the practical usefulness of the multiple decision criteria. It was agreed that the term *integrated assessment* was more appropriate than *weight-of-evidence* to describe the approach taken to classify substances that fell into the uncertainty range.

The Panel discussed when it was appropriate to rely on hypothesis testing (as opposed to decision criteria based on a cutoff SI value) to classify substances. The Panel commented that, in some cases, statistical significance might not indicate a biological effect. The Panel agreed with the language regarding hypothesis testing in the current ICCVAM LLNA Performance Standards (Appendix A - Section 3.0).

Public Comments:

At the conclusion of the Panel discussion, Dr. Luster called for public comments.

Dr. George De George, MB Research Labs
Dr. De George raised the following points:

- The data evaluated for the 1999 ICCVAM evaluation of the LLNA were statistically analyzed.
- As a result of that analysis, the optimum SI cutoff for a sensitizer was determined as 3.16.
- The Panel for the 1999 evaluation chose 3.0 as the SI cutoff to provide an added level of confidence.
- Routine statistical analysis of LLNA data to classify test substances was not recommended in the 1999 evaluation. In Dr. DeGeorge's opinion, the best reason to collect individual animal data was so that, in the future, studies could be done to determine an optimum method for hypothesis testing of LLNA data.
- Newer variant LLNA tests should be subjected to the same level (and not held to a higher level) of requirements for validation as the traditional LLNA.

Panel Conclusions and Recommendations:

At the conclusion of the public comments, Dr. Luster asked if the Panel was in agreement with the conclusions in the draft Panel Report as reflected in the updated presentation. The Panel approved unanimously.

Overview of the Revised Draft LLNA: BrdU-FC Test Method BRD and Revised Draft ICCVAM Test Method Recommendations

Dr. Luster called for Panel consideration of the LLNA: BrdU-FC test method. This test method measures bromodeoxyuridine (BrdU), a thymidine analog, instead of radioactive thymidine, incorporated into the DNA of proliferating lymphocytes, via flow cytometric analysis. The test method also allows for the measurement of immunophenotypic markers in the lymphocyte population, ostensibly aiding in discrimination between irritants and sensitizers.

Dr. George DeGeorge of MB Research Labs, Spinnerstown, PA (the test method developer) presented a synopsis of the test method to the Panel. In addition to a brief description of the test method protocol, Dr. DeGeorge made the following points:

- The test method protocol was based on the ICCVAM-recommended LLNA test method protocol, using SI $\geq$ 3.0 as the decision criterion for a sensitizer.
- Test substances were chosen to include those tested in the traditional LLNA.
- Guinea pig data and human results are considered less reliable.
- The LLNA: BrdU-FC uses lower doses of test substances than the traditional LLNA to avoid irritating concentrations.
- The LLNA: BrdU-FC makes correct calls for some substances for which the traditional LLNA does not.
- All of the data generated by MB Research Labs using the LLNA: BrdU-FC are available for review at the laboratory (although not all data are available electronically).
- MB Research Labs is currently attempting to find other laboratories interested in participating in an interlaboratory validation study.

Following Dr. De George's presentation, a Panelist asked the following questions:

- Does MB Research Labs conduct LLNA: BrdU-FC studies according to GLP? Dr. De George said yes.
- What is the treatment group size? Dr. DeGeorge responded that five animals per treatment group were used.
- Can measurement of ear swelling be added to any LLNA variant test method as an additional endpoint? Dr. DeGeorge replied that it could, and that it could help resolve which doses to test.

On behalf of NICEATM, Dr. Allen presented a summary of the revised draft LLNA: BrdU-FC BRD to the Panel. At the conclusion of Dr. Allen's presentation, Dr. DeGeorge pointed out that an in-house flow cytometer and trained operators weren't necessary to conduct the test method, because the lymphocytes were fixed as part of the test method protocol, and the flow cytometry analysis could be outsourced.

Dr. Jacobs then presented the revised draft ICCVAM test method recommendations for the LLNA: BrdU-FC test method to the Panel.

Panel Evaluation:

Dr. Richmond presented the draft position developed by Evaluation Group B, which was charged with primary review of the LLNA: BrdU-FC test method, to the Panel.

The Panel agreed that the LLNA: BrdU-FC test method was mechanistically and functionally similar to the traditional LLNA, and the ICCVAM LLNA Performance Standards could be used to evaluate it. The Panel also concurred that the database of more than 45 representative test substances yielded adequate accuracy based on results from one laboratory, and that intralaboratory reproducibility also had been adequately demonstrated. However, the Panel agreed with the ICCVAM proposal to defer a formal recommendation on the validity of the LLNA: BrdU-FC until an independent audit of all data supporting the analysis has been conducted and until transferability has been demonstrated in an interlaboratory validation study. The Panel recommended that ICCVAM should work with NICEATM to support and facilitate the independent audit and interlaboratory validation study. The Panel recommended that upon completion of these tasks and determination of satisfactory data quality, power, and interlaboratory reproducibility, that the LLNA: BrdU-FC could be considered to have adequate validation and performance to support its consideration for regulatory use.

Much Panel discussion about the necessary statistical power of the test method occurred. Power is defined as the probability that the test method would determine that a test group showing a positive result is different from the negative control (i.e., that a sensitizer would be detected as such). Data presented to the Panel during their 2008 evaluation indicated that the test method would require nine animals per treatment group to achieve 95% power; the power with five animals per group was estimated at 80% in that evaluation. The Panel agreed that, before an interlaboratory validation study was begun, it should be verified that the LLNA: BrdU-FC test method has power at least equal to that of the traditional LLNA using five animals per treatment group.

Public Comments:

At the conclusion of the Panel discussion, Dr. Luster called for public comments.

Dr. George De George, MB Research Labs

Dr. De George raised the following points:

- Power calculations on a subset of the data are not as reliable as accuracy statistics calculated from the entire dataset for 45 chemicals.
- Power calculations are a new requirement for validation, and not contained in the ICCVAM LLNA Performance standards.
- It was Dr. De George's opinion that it would be difficult, if not impossible, to get three qualified testing laboratories to participate in an interlaboratory validation study.

Panel Conclusions and Recommendations:

Subsequent to the public comments, the Panel commented that the flow cytometric analysis for samples from all three laboratories in an interlaboratory study could be done at MB Research Labs. Power calculations could be done by NICEATM on the most recent data generated by the LLNA: BrdU-FC test method.

The Panel decided to make a nomination to ICCVAM, with high priority, that NICEATM organize and supervise an interlaboratory validation study for the LLNA: BrdU-FC test method.

Dr. Luster asked if the Panel was in agreement with the conclusions in the draft Panel Report. The Panel approved unanimously.

Concluding Remarks

Dr. Luster, on behalf of the Panel, thanked the NICEATM-ICCVAM staff for their continued assistance during the review process and the Panel meeting. He also thanked Drs. Joanna Matheson and Abby Jacobs, the IWG co-chairs, and Dr. Marilyn Wind, ICCVAM Chair and IWG member, for the hard work they put into the project. Dr. Luster also thanked the Panel, the Evaluation Group Chairs, and the experts on the test methods, who presented them to the Panel.

Drs. Wind and Stokes thanked the Panel again for their hard work, thoughtful and objective deliberations, and advice. Dr. Stokes further thanked the invited test method developers for their excellent summaries of their test method for the benefit of the Panel. Dr. Stokes concluded by saying he looked forward to further working with the Panel members to complete their Panel report.

Adjournment

Dr. Luster adjourned the Panel at 11:30 a.m., concluding the meeting.

William S. Stokes, D.V.M., D.A.C.L.A.M.
NIEHS
P.O. Box 12233
Mail Stop: K2-16
Research Triangle Park, NC 27709

Dear Dr. Stokes,

The Meeting Summary Minutes, Independent Scientific Peer Review Panel Meeting, Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Updated Evaluation of the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA), accurately summarizes the Peer Review Panel meeting of April 28-29, 2009, in Bethesda, MD.

Sincerely,

Signature	Printed Name	Date
	MICHAEL LUSTER	8/21/09

Appendix D4

Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products

The full document is available electronically on the enclosed CD-ROM or at:
http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2009.pdf

The document is also available on request from NICEATM:

NICEATM
National Institute of Environmental Health Sciences
P.O. Box 1233, MD K2-16
Research Triangle Park, NC 27709 USA
Telephone: 919-541-2384 Fax: 919-541-0947
E-mail: niceatm@niehs.nih.gov

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Independent Scientific Peer Review Panel Report:

Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products

June 2009

Interagency Coordinating Committee on the Validation of Alternative Methods

National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

National Institute of Environmental Health Sciences
National Institutes of Health
U.S. Public Health Service
Department of Health and Human Services

National Toxicology Program
P.O. Box 12233
Research Triangle Park, NC 27709

This document is available electronically at:
http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2009.pdf

The findings and conclusions of this report are those of the Independent Scientific Peer Review Panel and should not be construed to represent the official views of ICCVAM or its member agencies.

When referencing this document, please cite as follows:

Interagency Coordinating Committee on the Validation of Alternative Methods. 2009. Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products. Research Triangle Park, NC: National Institute of Environmental Health Sciences.

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List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
ANOVA	Analysis of variance
AOO	Acetone: olive oil (4:1)
ATP	Adenosine triphosphate
BIBRA	British Industrial Biomedical Research Association
BRD	Background review document
BrdU	Bromodeoxyuridine
CV	Coefficient of variation
DNCB	2,4-Dinitrochlorobenzene
EC2	Estimated concentration of a substance needed to produce a stimulation index of 2 (value is expressed as a percentage)
EC2.5	Estimated concentration of a substance needed to produce a stimulation index of 2.5 (value is expressed as a percentage)
EC3	Estimated concentration of a substance needed to produce a stimulation index of 3 (value is expressed as a percentage)
ECt	Estimated concentration of a substance needed to produce a stimulation index that is indicative of a positive response (value is expressed as a percentage)
ECVAM	European Centre for the Validation of Alternative Methods
ELISA	Enzyme-linked immunosorbent assay
eLLNA: BrdU-FC	Enhanced LLNA: BrdU detected by flow cytometry
EPA	U.S. Environmental Protection Agency
ESAC	ECVAM Scientific Advisory Committee
E.U.	European Union
FR	*Federal Register*
GLP	Good Laboratory Practice
GPMT	Guinea pig maximization test
HCA	Hexyl cinnamic aldehyde
HMT	Human maximization test
HRIPT	Human repeat insult patch test
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ISO	International Organization for Standardization
IWG	ICCVAM Immunotoxicity Working Group
JSAAE	Japanese Society for Alternatives to Animal Experiments
LLNA	Murine local lymph node assay

LLNA: BrdU-ELISA	LLNA: BrdU detected by ELISA
LLNA: BrdU-FC	LLNA: BrdU detected by flow cytometry
LLNA: DA	LLNA: Daicel adenosine triphosphate
MRC	U.K. Medical Research Council
NAS	National Academy of Sciences
NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences
NIH	National Institutes of Health
NIOSH	National Institute of Occupational Safety and Health
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
QSAR	Quantitative structure–activity relationship
REACH	Registration, Evaluation, and Authorisation of Chemicals
RIFM	Research Institute for Fragrance Materials
rLLNA	Reduced LLNA
SACATM	Scientific Advisory Committee for the Validation of Alternative Toxicological Methods
SD	Standard deviation
SI	Stimulation index
SLS	Sodium lauryl sulfate
SOT	Society of Toxicology
UCSF	University of California, San Francisco

Members of the Independent Scientific Peer Review Panel

Michael Luster, Ph.D. (Panel Chair), Senior Consultant to the National Institute of Occupational Safety and Health (NIOSH) Health Effects Laboratory, Morgantown, WV

Nathalie Alépée, Ph.D., Scientific Coordinator on Alternatives Methods in Life Science, L'Oréal Research and Development, Aulnay sous Bois, France

Anne Marie Api, Ph.D., Vice President, Human Health Sciences, Research Institute for Fragrance Materials, Woodcliff Lake, NJ

Nancy Flournoy, M.S., Ph.D. (Biostatistician), Professor and Chair, Dept. of Mathematics & Statistics, University of Missouri – Columbia, Columbia, MO

Dagmar Jírová, M.D., Ph.D., Toxicologist, Research Manager, Head of Reference Center for Cosmetics, Head of Reference Laboratory for Experimental Immunotoxicology, National Institute of Public Health, Czech Republic

David Lovell, Ph.D. (Biostatistician), Reader in Medical Statistics, Postgraduate Medical School, University of Surrey, Guildford, Surrey, U.K.

Howard Maibach, M.D., Professor, Department of Dermatology, University of California – San Francisco, San Francisco, CA

Michael Olson, Ph.D., Director of Occupational Toxicology, Corporate Environment Health and Safety, GlaxoSmithKline, Research Triangle Park, NC

Raymond Pieters, Ph.D.,[1] Associate Professor, Immunotoxicology Group Leader, Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands

Jean Regal, Ph.D., Professor, Department of Pharmacology, University of Minnesota Medical School, Duluth, MN

Jonathan Richmond, MB ChB, FRCSEd, Head of Division, Animals Scientific Procedures Division, Home Office, London, U.K.

Peter Theran, V.M.D., Massachusetts Society for the Prevention of Cruelty to Animals, Novato, CA

Stephen Ullrich, Ph.D., Dallas/Ft. Worth Living Legends Professor & Professor of Immunology, Postgraduate School of Biomedical Science, University of Texas M.D. Anderson Cancer Center, Houston, TX

Michael Woolhiser, Ph.D., Science and Technology Leader, Toxicology and Environmental Research and Consulting, The Dow Chemical Company, Midland, MI

Takahiko Yoshida, M.D., Ph.D., Professor, Department of Health Science, Asahikawa Medical College, Hokkaido, Japan

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[1] Dr. Pieters was unable to attend the public meeting on April 28-29, 2009. However, he was involved in the review of the revised draft background review documents and the revised draft Addendum and concurs with the conclusions and recommendations included in this report.

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Preface

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended the murine local lymph node assay (LLNA) to U.S. Federal agencies as a valid substitute for currently accepted guinea pig test methods to assess the allergic contact dermatitis potential of many, but not all, types of substances. The recommendation was based on a comprehensive evaluation of the validation status of the LLNA that included an assessment by an international independent scientific peer review panel (hereafter, Panel). The LLNA was subsequently incorporated into national and international test guidelines for the assessment of skin sensitization (OECD 2002; ISO 2002; EPA 2003). (This LLNA will be referred to hereafter as the "traditional" LLNA.)

In January 2007, the U.S. Consumer Product Safety Commission formally requested that ICCVAM assess the validation status of:[2]

- The traditional LLNA as a stand-alone assay for potency determinations (including severity) for the purpose of hazard classification
- Three modifications of the traditional LLNA not requiring the use of radioactive materials
- The reduced LLNA (rLLNA; also referred to as the LLNA limit dose procedure)
- The ability of the traditional LLNA to test mixtures, metals, and aqueous solutions (i.e., to re-evaluate the applicability domain for the traditional LLNA)

The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), in coordination with ICCVAM and the ICCVAM Immunotoxicity Working Group (IWG), prepared comprehensive draft background review documents (BRDs) for each modified version of the traditional LLNA test method being evaluated, as well as a draft applicability domain addendum to the final BRD published previously on the traditional LLNA. In addition, ICCVAM developed draft LLNA performance standards intended for use in validating alternative test methods that are functionally and mechanistically similar to the traditional LLNA. Finally, ICCVAM, based on the information contained in each of the draft BRDs and the draft addendum, developed draft test method recommendations.

The supporting documents and the draft ICCVAM recommendations were provided to a new international Panel for an independent scientific review. This Panel met in public session in

[2] The U.S. Consumer Product Safety Commission nomination can be obtained at: http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf.

March 2008.[3] Subsequent to the Panel review, finalized recommended performance standards for the LLNA and ICCVAM recommendations for the rLLNA were published.[4] The final documents considered the comments of the Panel, the public, and ICCVAM's scientific advisory panel.

The Panel concluded in March 2008 that more information and data were required for the three modified nonradioactive LLNA test methods before recommendations could be made regarding their use for regulatory safety testing (ICCVAM 2008). Similarly, the Panel concluded that more data would be needed before a recommendation on the usefulness and limitations of the current applicability domain of the traditional LLNA could be made. Subsequent to the Panel meeting, NICEATM received additional LLNA data for pesticide formulations and other products, as well as new data for the three modified nonradioactive LLNA test methods.

Using the additional information and working in coordination with the IWG, NICEATM revised the BRDs for each of these modified test methods and new applications of the LLNA. The revised draft BRDs provide the data and analyses supporting the scientific validity of the modified test methods and proposed applications. ICCVAM also prepared revised draft test method recommendations regarding proposed usefulness and limitations, standardized protocols, and future studies.

The revised draft BRDs, the revised draft applicability domain addendum, and revised draft ICCVAM recommendations were provided to the Panel for independent scientific review. In addition, NICEATM announced the availability of these documents on the NICEATM – ICCVAM website for public comment in a *Federal Register* (FR) notice (74 FR 8974) and via the ICCVAM email list. The FR notice also announced the public Panel meeting, to be convened at the National Institutes of Health in Bethesda, Maryland, on April 28 – 29, 2009.

The Panel was charged with:

- Reviewing each revised draft BRD and the revised draft addendum for completeness, and identifying any errors or omissions of existing relevant data or information
- Evaluating the information in each revised draft BRD and the revised draft addendum to determine the extent to which each of the applicable criteria for validation and acceptance of toxicological test methods (ICCVAM 2003) had

[3] The conclusions and recommendations of the Panel are included in its report, which is available at: http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf.

[4] The *Recommended LLNA Performance Standards* document is available at: http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna-ps/LLNAPerfStds.pdf; the ICCVAM

been appropriately addressed for the recommended use of the new versions and applications of the traditional LLNA

- Considering the ICCVAM revised draft test method recommendations for the following, and commenting on the extent to which they are supported by the information provided in the revised draft BRDs and the revised draft addendum:
  - Proposed test method uses
  - Proposed recommended standardized protocols
  - Proposed test method performance standards
  - Proposed additional studies

During its public meeting in April 2009, the Panel discussed each charge, listened to public comments, and developed conclusions and recommendations for ICCVAM. The Panel emphasizes that it was asked to consider two overall questions. The Panel was to consider: (1) whether the validation status of each of the above proposed modifications or alternative uses of the LLNA had been adequately characterized for its intended purpose according to established ICCVAM validation criteria,[5] and (2) whether proposed modifications or alternative uses of the LLNA are sufficiently accurate and reliable to be used for the identification of sensitizing substances and nonsensitizing substances in place of the traditional LLNA procedure.

This report details the Panel's independent conclusions and recommendations. ICCVAM will consider this report, along with all relevant public comments, as it develops final test method recommendations. The final ICCVAM test method recommendations will be forwarded to U.S. Federal agencies for their consideration in accordance with the ICCVAM Authorization Act of 2000 (Public Law 106-545).

The Panel gratefully acknowledges the efforts of NICEATM staff in coordinating the logistics of the peer review Panel meeting and in preparing materials for the Panel's review. The Panel also thanks each of the test method developers, Drs. George DeGeorge (LLNA: bromodeoxyuridine detected by flow cytometry test method), Kenji Idehara (LLNA: Daicel adenosine triphosphate test method), and Masahiro Takeyoshi, (LLNA: bromodeoxyuridine detected by ELISA) for providing summaries and additional clarifications of the

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recommendations for the rLLNA are in the *ICCVAM Test Method Evaluation Report*, available at: http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNA-LD/TMER.pdf.

[5] ICCVAM validation criteria are detailed in the document, *Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods,* available at http://iccvam.niehs.nih.gov/docs/about_docs/validate.pdf.

nonradioactive test methods under review. Finally, as Panel Chair, I thank each Panel member for her or his thoughtful and objective review of these LLNA-related activities.

Michael Luster, Ph.D.
Chair, LLNA Peer Review Panel
June 2009

Executive Summary

This report describes the conclusions and recommendations of an international independent scientific peer review panel (hereafter, Panel). This Panel was charged by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) with evaluating the validation status of new versions and applications of the murine local lymph node assay (LLNA) for assessing the allergic contact dermatitis (ACD) potential of chemicals and products. The LLNA which was first evaluated in 1999 by ICCVAM is hereafter referred to as the "traditional LLNA" to distinguish it from other versions considered by the Panel. The new versions and applications considered include:

- The application of the traditional LLNA for evaluating pesticide formulations and other products, metals, and substances in aqueous solutions
- Three modified versions of the traditional LLNA not requiring the use of radioactive markers:
  - LLNA: DA (LLNA: Daicel adenosine triphosphate)
  - LLNA: BrdU-FC (LLNA: bromodeoxyuridine detected by flow cytometry)
  - LLNA: BrdU-ELISA (LLNA: bromodeoxyuridine detected by ELISA)

Nonradioactive LLNA Protocol – The LLNA: DA Test Method

The Panel concluded that the available data and performance support the revised draft ICCVAM recommendations on usefulness and limitations for the LLNA: DA test method. They agreed that the test method could be used for identifying substances as potential skin sensitizers and nonsensitizers. On the basis of the available data, accuracy is optimized if a stimulation index (SI) ≥ 2.5 is used to identify sensitizers, and an SI ≤ 1.7 is used to identify nonsensitizers. A limitation of the LLNA: DA involves the indeterminate identification of substances with SI values between 1.7 and 2.5 (exclusive). Thus, when an SI between 1.7 and 2.5 is obtained in the LLNA: DA, users should carefully interpret the results in an integrated decision strategy in conjunction with all available and relevant information (e.g., dose response information, statistical analyses, peptide-binding activity, molecular weight, results from related chemicals, other testing data) to determine if there is adequate information for a definitive skin sensitization identification or if additional testing is necessary. The Panel noted that because the decision criteria chosen to identify sensitizers and nonsensitizers were based on a post hoc analysis, prospective testing with the test method might affect the proposed model. For this reason, data generated should be routinely evaluated to determine if the proposed model is still optimal with regard to the decision criteria. Even with these limitations, the LLNA: DA provides opportunities to reduce animal usage (e.g., use of guinea

pigs) in those regions in which guinea pig tests rather than the traditional LLNA are performed because radioisotope use is not permitted. In addition, the use of two decision criteria allows for a more definitive identification of sensitizers and nonsensitizers, which also provides animal welfare benefits by reducing further tests that might be required in instances where the hazard classification of a substance is not as clear.

The revised draft LLNA: DA background review document (BRD) was compiled to provide a comprehensive review of available data and information evaluating the usefulness and limitations of the LLNA: DA test method to assess the ACD-inducing potential of chemicals and other products. The Panel evaluated the revised draft BRD for completeness, errors, and omissions, and recommended that its suggestions/corrections relating to general, statistical, and specific editorial issues be incorporated into future revisions.

The Panel agreed that the data supported the revised draft ICCVAM recommendations for the proposed standardized protocol for the LLNA: DA. The recommendations for maintaining a positive control database reflect current evidence and best practice. The Panel agreed that four animals per dose group should be recommended for the LLNA: DA.

The Panel considered the substances tested in the LLNA: DA to be representative of a sufficient range of chemicals expected to be tested for skin sensitization potential, and concluded that the accuracy analysis had made appropriate comparisons to the traditional LLNA, guinea pig tests, and human data/experience. The Panel indicated that the number of substances in the range of uncertainty was too few to determine if specific characteristics (e.g., chemical class, physical form, molecular weight, peptide reactivity, etc.) associated with those substances could be used for definitive skin sensitization identification.

With regard to test method reliability, the Panel concluded that the interlaboratory reproducibility of the LLNA: DA had been adequately evaluated. The Panel noted that five of the 10 laboratories that participated in the first phase of the interlaboratory validation study exceeded the performance standards' acceptable range for ECt values (estimated concentration of a substance needed to produce an SI that is indicative of a positive response) for 2,4-dinitrochlorobenzene (DNCB). The Panel indicated that this was understandable since DNCB is a strong sensitizer and the LLNA: DA has a different dosing regimen and time course than the traditional LLNA, which might extend into the elicitation phase of skin sensitization. However, all the laboratories that participated in the first and second phase of the interlaboratory validation study obtained EC2.5 values (estimated concentration of a substance needed to produce an SI of 2.5) within the concentration range indicated for hexyl cinnamic aldehyde (HCA), which documents the test method's favorable reproducibility and performance.

The Panel stated that the available data supported the revised draft ICCVAM recommendations for the LLNA: DA in terms of future studies, which included performing more LLNA: DA studies on metals, irritants, and formulations with comparative traditional LLNA, guinea pig, and human data. Regarding irritants, the proposed future studies might help explain why results obtained using the LLNA: DA were discordant with the traditional LLNA and may even provide general insight into the problematic nature of discriminating irritants in the LLNA. The Panel also recommended that additional decision criteria and guidance should be identified for substances with SI greater than 1.7 but less than 2.5, and that the additional decision criteria be reassessed as additional discriminators and data become available (e.g., high-quality human ACD data). The Panel recommended that a protocol for defining and reevaluating the SI decision criteria for sensitizers and nonsensitizers be developed. Further, future interlaboratory validation studies should simultaneously evaluate intralaboratory reproducibility, using the appropriate statistics, to evaluate variation both within a laboratory and between laboratories. Additionally, the Panel strongly recommended that a statistician actively participate in the preparation of future BRDs and formulation of ICCVAM recommendations.

The Panel disagreed with the revised draft ICCVAM recommendation that separate performance standards be developed to assess modified versions of the LLNA: DA test method. Although the test methods differ in the dosing regimen and in the timing of the assay, the Panel viewed the LLNA: DA as mechanistically similar to the traditional LLNA, in that both methods measure cellular stimulation in the draining lymph nodes. Consequently, the Panel concluded that the ICCVAM-recommended LLNA performance standards (ICCVAM 2009) are applicable to the LLNA: DA as a mechanistically and functionally similar test method. Generally, the Panel viewed the difference in treatment schedule between the LLNA: DA and the traditional LLNA to be potentially significant if the LLNA: DA test progressed through the elicitation phase of skin sensitization, which is associated with a localized skin reaction. Thus, the Panel was concerned that if the duration of the test involved the elicitation phase of ACD development, this would produce undue discomfort and distress in the animals. The Panel also recommended that the test method developer (Daicel Chemical Industries, Ltd.) justify the use of 1% sodium lauryl sulfate (SLS) (i.e., determine whether the 1% SLS pretreatment is necessary).

Nonradioactive LLNA Protocol – The LLNA: BrdU-FC Test Method

The Panel concluded that the data and test method performance of the LLNA: BrdU-FC supported the revised draft ICCVAM recommendations that the test method may be useful for identifying substances as potential skin sensitizers or nonsensitizers, and agreed that formal recommendations should be deferred until original study records are received for an

independent audit and interlaboratory transferability and reproducibility have been assessed. The final test method recommendations should highlight those items of highest priority for further validation consideration: (1) a review of the original data at the individual animal level with appropriate positive and negative controls, (2) an evaluation, based on the data from the intralaboratory study data, of the minimum number of animals required per test group to ensure test performance is as good as or better than the traditional LLNA, then (3) an interlaboratory reproducibility study conducted and evaluated according to the specifications in the ICCVAM-recommended LLNA performance standards (ICCVAM 2009) and with appropriate quality control systems. The Panel agreed that, subsequently, less critical items (e.g., methodological specifics, immunophenotypic endpoints, alternative decision criteria for identifying materials as sensitizers and nonsensitizers) should then be evaluated.

The revised draft LLNA: BrdU-FC BRD was compiled to provide a comprehensive review of available data and information evaluating the usefulness and limitations of the LLNA: BrdU-FC test method to assess the ACD-inducing potential of chemicals and other products. The Panel evaluated the revised draft BRD for completeness, errors, and omissions, and recommended that its recommendations/corrections relating to general, statistical, and specific editorial issues be incorporated into future revisions.

The Panel agreed that the available data supported the revised draft ICCVAM recommendations for the proposed test method protocol for the LLNA: BrdU-FC procedure. Also, revised power calculations should be performed using the data provided for the intralaboratory performance to determine the minimum group size required to provide a level of test performance equivalent to or better than the traditional LLNA. The minimum group size in the protocol should then be adjusted, if necessary. The ICCVAM recommendation for maintaining a positive control database reflects current evidence and best practice. The Panel considered the measurement of ear swelling and the use of immunophenotypic markers as potentially valuable adjuncts to the traditional LLNA and other modified LLNA protocols.

The Panel noted that since the 2008 Panel evaluation no new data for additional test substances were added to the analyses in the revised draft BRD, although new data for intralaboratory reproducibility were properly integrated into the assessment. As such, similar to 2008, the substances tested in the LLNA: BrdU-FC seemed representative of a sufficient range of chemical classes and physical chemical properties, and thus the test method appeared applicable to many of the types of chemicals and products that are typically tested for skin sensitization potential. The results of the revised concordance assessments of the LLNA: BrdU-FC against the traditional LLNA test method suggest that the LLNA: BrdU-FC (as performed at the originating facility) can be developed as a reliable alternative to the

traditional LLNA, with the same applicability domain. Both the LLNA: BrdU-FC and the eLLNA: BrdU-FC ("enhanced" LLNA: BrdU-FC), on the basis of the information available, performed equally well compared with the traditional LLNA in a single laboratory.

The Panel concluded that compared to the 2008 review, intralaboratory reproducibility was adequately assessed and fit for the intended purpose. This was based on additional studies submitted for HCA and DNCB. The Panel agreed that the assessment of interlaboratory reproducibility described in the ICCVAM-recommended LLNA performance standards (ICCVAM 2009) can be appropriately applied to the LLNA: BrdU-FC test method.

The Panel affirmed that the revised draft ICCVAM recommendations for future studies highlighted the unanswered questions raised by the available data set. The Panel specifically recommended: (1) that an independent audit of the original data should be performed to establish the validity of the data relied upon in the revised draft BRD, (2) that revised power calculations should be performed using the data provided for the intralaboratory validation so that the number of animals needed to provide performance equivalent to, or better than, the traditional LLNA can be determined, (3) that an interlaboratory study is an absolute requirement for validation to determine the transferability and reliability of the test method when used in different laboratories, (4) that alternate prediction models (e.g., multiple SIs similar to those recommended for the LLNA: DA and LLNA: BrdU-ELISA test methods) should be considered, and (5) that the ICCVAM-recommended LLNA performance standards (ICCVAM 2009) should be followed in this future work. The Panel recommended that ICCVAM should work with NICEATM to support and facilitate these activities. The Panel also considered that an emphasis should be given to the use of ear swelling measurements to identify local irritants as a means of improving the traditional LLNA and modified LLNA test methods. This is particularly relevant when considering the challenges associated with discriminating irritants from sensitizers in the LLNA and ultimately emphasizes the need to better understand the correlation between mouse ear data and human data/experience.

It is the view of the Panel that this test method can be considered to have been scientifically validated and to be ready for regulatory consideration if the following requirements are satisfactorily met: (1) an independent data audit should be conducted confirming the acceptable quality of the data relied upon in the revised draft BRD, (2) a revised evaluation of the minimum number of animals required should be conducted; then, if n = 4 or 5 yields statistical power that is equivalent to or better than the traditional LLNA, an interlaboratory evaluation should be performed using the test, (3) the interlaboratory study should produce results that satisfy the requirements in the ICCVAM-recommended LLNA performance standards (ICCVAM 2009).

The Panel considered the LLNA: BrdU-FC and the traditional LLNA to be mechanistically and functionally similar. Thus, the studies proposed by the ICCVAM-recommended LLNA performance standards are sufficient to establish the intra- and interlaboratory performance of the LLNA: BrdU-FC. The Panel commented that for regulatory data submissions, a laboratory (either with flow cytometry experience and/or following training and certification of personnel) should demonstrate proficiency by repeating the evaluation of the same substance (i.e., four independent tests) to allow an assessment of intralaboratory reproducibility before using the test for regulatory purposes. Results should be evaluated for both a known strong and known moderate sensitizer (i.e., DNCB and HCA, respectively). The inclusion of a known, reproducible weak sensitizer and a negative control is also essential to confirm that the full range of appropriate responses can be reproduced.

Additional considerations would include development of a standard test method protocol, standard operating procedure, and other documentation, and adherence to recognized quality assurance/quality control systems for flow cytometry and associated data acquisition equipment.

Nonradioactive LLNA Protocol – The LLNA: BrdU-ELISA Test Method

The Panel concluded that the data and performance for the LLNA: BrdU-ELISA test method supported the revised draft ICCVAM recommendations that it can be used for identifying substances as potential skin sensitizers and nonsensitizers. An SI $\geq$ 2.0 should be used to identify substances as sensitizers and SI < 1.3 should be used to identify nonsensitizers. A limitation of the LLNA: BrdU-ELISA involves the indeterminate identification of substances that produce an SI greater than or equal to 1.3 but less than 2.0. When such a result is obtained in the LLNA: BrdU-ELISA, users should carefully interpret the results in an integrated decision strategy in conjunction with all available and relevant information (e.g., dose response information, statistical analyses, peptide-binding activity, molecular weight, results from related chemicals, other testing data) to determine if there is adequate information for definitive skin sensitization identification or if additional testing is necessary. The Panel noted that because the decision criteria chosen to identify sensitizers and nonsensitizers were based on post hoc analysis, prospective testing with the test method might affect the proposed model. For this reason, data generated should be routinely evaluated to determine if the proposed model is still optimal with regard to the decision criteria. Even with these limitations, the LLNA: BrdU-ELISA provides opportunities to reduce animal usage (e.g., use of guinea pigs) in those regions that are not permitted to use radioisotopes and thus perform guinea pig tests rather than the traditional LLNA. In addition, using two decision criteria allows for a more definitive identification of sensitizers and

nonsensitizers, which also provides animal welfare benefits by reducing further tests that might be required in instances where the hazard classification of a substance is not as clear.

The revised draft LLNA: BrdU-ELISA BRD was compiled to provide a comprehensive review of available data and information evaluating the usefulness and limitations of the LLNA: BrdU-ELISA test method to assess the ACD-inducing potential of chemicals and other products. The Panel evaluated the draft BRD for completeness, errors, and omissions and recommended that its suggestions/corrections relating to general, statistical and specific editorial issues be incorporated into the final document.

The Panel agreed that the available data supported the revised draft ICCVAM recommendations for the proposed standardized test method protocol for the LLNA: BrdU-ELISA test method. The recommendations for maintaining a positive control database reflect current evidence and best practice. The Panel agreed that four animals per dose group should be recommended for the LLNA: BrdU-ELISA.

The Panel considered the database of substances tested in the LLNA: BrdU-ELISA to be representative of a sufficient range of chemicals expected to be tested for skin sensitization potential, and concluded that the accuracy analysis had made appropriate comparisons to the traditional LLNA, guinea pig tests, and human data/experience. The Panel indicated that the number of substances in the range of uncertainty (i.e., $1.3 \leq SI < 2.0$) was too few to determine if specific characteristics (e.g., chemical class, physical form, molecular weight, peptide reactivity, etc.) associated with those substances could be used for definitive skin sensitization identification.

In 2008, the Panel did not find sufficient power for using $SI \geq 1.3$ as the decision criterion. Even with a group size of eight animals, the power was only 50% (ICCVAM 2008). Power calculations might be necessary to determine if the sample size used is sufficient for those substances that are not definitively identified as sensitizers or nonsensitizers (i.e., substances in the range of uncertainty of $1.3 \leq SI < 2.0$).

With regard to test method reliability, the Panel concluded that the interlaboratory reproducibility had been adequately evaluated and that the test is reproducible. Considering that the radioisotope measurement in the traditional LLNA is more sensitive than the technique for the LLNA: BrdU-ELISA, and that the analysis of EC3 values (estimated concentration of a substance needed to produce a stimulation index of 3) in the traditional LLNA was based on a larger dataset, it is appropriate to adjust the acceptability range of the two positive control substances tested, dependent on the method used for measurement of the endpoint. Although the qualitative performance was acceptable in the interlaboratory study, the quantitative data for two of the laboratories suggests a relatively high degree of variability, which justifies the routine use of appropriate positive and negative controls.

The Panel stated that the available data supported the revised draft ICCVAM recommendations for the LLNA: BrdU-ELISA in terms of future studies, which included performing more LLNA: BrdU-ELISA studies on metals, irritants, and formulations with comparative traditional LLNA, guinea pig, and human data. Regarding irritants, the proposed future studies might help explain why results obtained using the LLNA: BrdU-ELISA and traditional LLNA were discordant, and further address the general challenge of discriminating irritants in the traditional LLNA itself. The Panel also recommended that additional decision criteria and guidance should be identified for substances that produce an SI greater than or equal to 1.3 but less than 2.0, and that the additional decision criteria be reassessed as additional discriminators and data become available (e.g., high-quality human ACD data). The Panel recommended that a protocol for defining and reevaluating the SI decision criteria for sensitizers and nonsensitizers be developed. Further, future interlaboratory validation studies should simultaneously evaluate intralaboratory reproducibility, using the appropriate statistics, to evaluate variation both within a laboratory and between laboratories. As stated previously, the Panel strongly recommended that a statistician actively participate in the preparation of future BRDs and formulation of ICCVAM recommendations.

The Panel agreed with the revised draft ICCVAM recommendation that separate performance standards should not be developed to assess modified versions of the LLNA: BrdU-ELISA test method. The LLNA: BrdU-ELISA is mechanistically and functionally similar to the traditional LLNA, such that the ICCVAM-recommended LLNA performance standards (ICCVAM 2009) could be used to evaluate future modifications of the LLNA: BrdU-ELISA.

LLNA for Testing Pesticide Formulations and Other Products, Aqueous Solutions, and Metals

The Panel comprises experts with knowledge in the evaluation of a range of test materials, but it is by no means expert in all of the product classes for which skin sensitization potential should be evaluated. The Panel also acknowledges that information and data gaps exist which prevent a full understanding of ACD epidemiology in humans. The test materials for which data are provided in the revised draft Addendum cover only a subset of the active ingredients used in each of the relevant product classes, and their frequency of use within those product classes is not noted in the revised draft Addendum. The Panel recommends that Federal agencies considering the results of this validation process assess how representative the test materials and findings in the revised draft Addendum are relative to substances of interest. In particular, the agencies should assess the chemical classes used in, and the range of biological effects of, the materials and products in which they have an interest.

The revised draft ICCVAM recommendations state that, although the database is limited, the traditional LLNA appears to be useful for evaluating substances tested in aqueous solutions or pesticide formulations provided the potential for overclassification (i.e., false positives) is not a limitation. The Panel agreed with these revised draft ICCVAM recommendations noting that the high rate of false positive substances may be inherent to the product and/or chemical class, testing of substances at concentrations that produced skin irritation, and to the fact that the LLNA detects the induction phase of skin sensitization. Furthermore, where comparative data were available, the LLNA identified more sensitizers than did guinea pig tests (predominantly Buehler tests which are considered to be less sensitive than the guinea pig maximization test [Basketter et al. 1993; Frankild et al. 2000]) but missed no materials that the guinea pig tests classified as sensitizers.

The Panel further suggested that, unless there are unique physiochemical properties associated with a material that might affect its ability to interact with immune processes, it should be a candidate for LLNA testing. An example of a material class that may possess such unique properties is some nanomaterials that are incapable of recognition by dendritic cells. Along these lines, the Panel also disagreed with the revised draft ICCVAM recommendation that a definitive recommendation on the usefulness of the LLNA for testing natural complex substances and dyes could not be made until more data were accrued. The Panel considered these classes of materials suitable for testing in the LLNA unless there are unique physiochemical properties associated with these materials that might affect their ability to interact with immune processes.

The Panel expressed a strong desire to avoid revalidation of the LLNA for new classes/types of test substances unless there is a biologically-based rationale. For new classes of test materials (e.g., nanomaterials), an integrated assessment of all available and relevant information should be conducted. This should include computer-assisted structure-activity relationships, prediction/measurement of biotransformation to potential reactive species, and possibly peptide, protein, or lipid binding. The Panel agreed that if any variant of the LLNA is validated for use to test novel classes, then the findings should be relevant to the family of validated LLNA tests and that similar uncertainties would surround the use of guinea pig models to evaluate novel classes of test materials.

The revised draft Addendum to the original validation report for the traditional LLNA (ICCVAM 1999) provided a comprehensive review of currently available data and information for evaluating the usefulness and limitations of the traditional LLNA for assessing the skin sensitization potential of pesticide formulations and other products, substances tested in aqueous solutions, and metals. The Panel evaluated the revised draft Addendum for completeness, errors, and omissions and concluded that there were no apparent errors. However, a Panel member did

note during the public meeting an omission regarding the natural complex substances; the relationship between the LLNA, guinea pig, and human data for major constituents (substances constituting at least 70%) of some of the natural complex substances and the LLNA results of the natural complex substances themselves was omitted. The Panel recommended that its suggestions/corrections relating to general, statistical, and specific editorial issues be incorporated into future revisions.

The Panel stated in its 2008 review (ICCVAM 2008) that the term *mixtures* was used too broadly (i.e., can represent an infinite number of materials), and this concern was addressed in the revised draft Addendum by dividing the substances considered into pesticide formulations, dyes, natural complex substances, and substances tested in aqueous solutions (this group included pesticide formulations tested in aqueous solutions), and analyzing the data for each group separately. The Panel agreed that the terms used to classify information submitted for the revised analysis are sensible and help to divide the dataset into useful categories for analysis, and that the product categories selected fit well with the nature and range of materials in the database. Such categories indicate classes of materials for which there exist, or do not exist, LLNA data and thus provide useful information for industry and regulatory agencies.

The Panel noted that the revised draft Addendum does not consider many classes of formulations to which humans may be exposed, by intention or by accident, such as: metalworking fluids, fuels, petroleum products used as lubricants, detergents and other cleaning agents, enzymes used in cleaning products, chemical household products, chemical (low molecular weight) pharmaceutical products, medical device materials (chemically characterized extracts), and nanomaterials (e.g., titanium oxide). Available data for substances within these classes may prove informative for human health.

Regarding pesticide formulations, the Panel concluded that the performance characteristics, reproducibility, and reliability of the LLNA had been adequately assessed and that the methods of data analysis were appropriate. The Panel indicated that the analysis for dyes, natural complex substances, and substances tested in aqueous solutions reflected the available information and the appropriate concordance statistics.

With regard to future studies, the Panel agreed with the ICCVAM recommendation for continued accumulation of information in the targeted areas. The Panel also indicated that solubility data should ideally be provided so that thermodynamic activity can be computed and compared to maximum theoretical percutaneous penetration. This information should be considered when comparing the data from LLNA studies in lipophilic delivery systems compared to that in aqueous systems. The Panel also suggested that, before additional animal testing is conducted, consideration should be given to product use and whether this renders a need to test the substance for skin sensitization potential.

Appendix A

Peer Review Panel Member Biosketches

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Panel Member Biosketches

Nathalie Alépée, Ph.D.

Dr. Alépée performed research leading to a Ph.D. in Medical Virology and Microbiology at the Centre National de la Recherche Scientifique institute, Gif sur Yvette, France. She is currently the scientific coordinator on Alternatives Methods in Life Science Department at L'Oréal Research and Development, Aulnay sous Bois, France. She is the L'Oréal representative to the European Partnership on Alternative to Animal Testing, and serves on two working groups: Identification of Opportunities, Including R&D (working group 2), and Validation and Acceptance (working group 5). She is also the representative in the eye irritation working group to the European Cosmetics Association and in the French Groupement d'Intérêt Scientifique Platform on Alternatives. She has served on the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee (ESAC), representing the European Federation of Pharmaceutical Industries Associations, and was nominated as Organisation for Economic Co-operation and Development expert for eye and skin irritation. As a manager in Investigative Toxicology with Pfizer Global Research and Development, Amboise, France, she implemented the murine local lymph node assay (LLNA) in the laboratory. She served as a peer reviewer of the reduced LLNA test protocol and prediction model for ESAC in 2007, and has been designated as an ESAC peer reviewer for ECVAM's performance standards for the standard LLNA.

Anne Marie Api, Ph.D.

Dr. Api received a Ph.D. from Aston University in Birmingham, England and is currently Vice President of Human Health Sciences at the Research Institute for Fragrance Materials (RIFM). She is responsible for the human health scientific program and for the investigation and initiation of new research and testing projects for RIFM. She is a member of 10 professional organizations, including the American Academy of Dermatology, American Contact Dermatitis Society, the European Society of Contact Dermatitis, and the Society of Investigative Dermatology. She participated in the World Health Organization International Workshop in Skin Sensitization in Chemical Risk Assessment held in Berlin, Germany, in 2006, and a BfR International Workshop on Contact Dermatitis in October 2008. She is author of over 100 publications and presentations relevant to dermatology and dermatotoxicology.

Nancy Flournoy, M.S., Ph.D.

Dr. Flournoy received B.S. and M.S. degrees in Biostatistics from the University of California at Los Angeles, and a Ph.D. in Biomathematics from the University of Washington. She is Professor and Chair of the Department of Statistics at the University of

Missouri. Her research interests include adaptive designs, bioinformatics, chemometrics, clinical trials, and environmetrics. She has an extensive list of edited volumes and papers on statistical theory, statistical genetics and immunology, epidemiology in immune-suppressed subjects, clinical trials for prevention and treatment of viral infection, transplantation biology and its effects on digestion, lungs, eyes, mouth, and central nervous system, optimization of statistical processing, and additional papers, interviews, and technical reports. She has editorial responsibilities for numerous statistical journals and serves on numerous advisory boards and nominating committees. She is a member and past Chair of the Council of Sections of the American Statistical Association, and served in various other statistical, medical and toxicological societies or programs as Chair or as a member of the Board of Directors. She is a former member of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM). She also served on the Expert Panels for the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) that evaluated the Revised Up-and-Down Procedure; the Current Validation Status of *In Vitro* Test Methods for Identifying Ocular Corrosives and Severe Irritants; and Five *In Vitro* Pyrogen Test Methods.

Dagmar Jírová, M.D., Ph.D.

Dr. Jírová received a Ph.D. from the Medical Faculty of Hygiene at Charles University in Prague. She is currently the Head of the Department of Toxicology and Veterinary Services and the Reference Center for Cosmetics at the National Institute of Public Health in the Czech Republic. Her main responsibilities include safety assessment of consumer products, particularly cosmetics and their ingredients, performance of toxicological methods *in vivo* in animals, human patch testing for local toxicity assessment, and introduction of *in vitro* techniques for screening of toxicological endpoints using cell and tissue cultures. She represents the Czech Republic in the Standing Committee on Cosmetics of the European Commission. She is an ESAC-ECVAM member and was involved in the Peer Review Panel for Skin Irritation Validation Study and LLNA test protocol and prediction model. She is author of more than 100 publications and presentations relevant to dermatotoxicology, including a recent presentation at the Sixth World Congress on Alternatives and Animal Use in the Life Sciences, held in Tokyo, 2007, titled “Comparison of Human Skin Irritation and Photoirritation Patch Test Data with Cellular *in vitro* Assays and Animal *in vivo* data”.

David Lovell, Ph.D., B.Sc. (Hons), F.S.S., FIBiol, CStat, CBiol

Dr. Lovell received a Ph.D. from the Department of Human Genetics and Biometry, University College, London. He is currently Reader in Medical Statistics at the Postgraduate Medical School at the University of Surrey. Previously, he was Associate Director and Head

of Biostatistics support to Clinical Pharmacogenomics at Pfizer Global Research and Development in Sandwich, Kent, providing data management and statistical support to pharmacogenetics and genomics. He joined Pfizer in 1999 as the Biometrics Head of Clinical Pharmacogenetics. Before joining Pfizer, Dr. Lovell was the Head of the Science Division at British Industrial Biological Research Association (BIBRA) International, Carshalton, which included Molecular Biology, Genetic Toxicology, Biostatistics and Computer Services. At BIBRA, Dr. Lovell managed the statistical and computing group providing specialized statistical support to BIBRA's Clinical Unit and contract research work. He conducted and managed research programs on genetics, statistics and quantitative risk assessment for the European Union and U.K. Government Departments. His research interests are the use of mathematical, statistical, and bioinformatic methods together with genetic models in the understanding of toxicological mechanisms and risk assessment problems. Dr. Lovell had previously been a Senior Research Officer with the U.K. Medical Research Council (MRC) Experimental Embryology and Teratology Unit, a visiting Postdoctoral Fellow at the U.S. National Institute of Environmental Health Sciences (NIEHS), a Geneticist at the MRC Laboratories, Carshalton, and a Research Assistant in Cytogenetics at Birmingham University. He has acted as a consultant to a number of organizations, has considerable experience of working with Regulatory Authorities, has many publications related to his work and has wide experience of making presentations to a wide range of audiences. He is a member of the Scientific Committee of the European Food Safety Authority, the U.K. Government's advisory Committees on Mutagenicity and Carcinogenicity of Chemicals in Food, Consumer Products and the Environment and the Independent Scientific Advisory Committee for the U.K. Medicines and Healthcare Products Regulatory Agency database research. He served on the NICEATM-ICCVAM Expert Panels that evaluated the Frog Embryo Teratogenesis Assay - Xenopus, *In Vitro* Test Methods for Identifying Ocular Corrosives and Severe Irritants, and Five *In Vitro* Pyrogen Test Methods.

Michael Luster, Ph.D.

Dr. Luster received a Ph.D. in Immunology from Loyola University of Chicago. He was formerly Chief, Toxicology and Molecular Biology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health (NIOSH), and currently serves as a senior advisor to the Director of the Health Effects Laboratories and the staff of Toxicology and Molecular Biology Branch at NIOSH. Program areas include neuroscience, dermatology, molecular carcinogenesis, molecular epidemiology, molecular toxicology, molecular epidemiology, and inflammation/immunotoxicology. In addition, Dr. Luster conducts basic and applied research in immunotoxicology including its application in risk assessment. Current research activities include molecular epidemiology studies of genetic polymorphism involved in workplace-related diseases and experimental studies involving

occupational allergic rhinitis. Dr. Luster is also working with various staff at the U.S. Environmental Protection Agency (EPA) through the Risk Assessment Forum to develop immunotoxicity testing guidelines. He also directed two studies for the NTP on the Toxicology and the Carcinogenesis of Promethazine and Ortho-phenylphenol, in 1990 and 1986, respectively. He is a co-author of over 300 publications in peer-reviewed journals.

Howard Maibach, M.D.

Dr. Maibach received an M.D. from Tulane University. He is currently a professor in the Department of Dermatology at the University of California, San Francisco (USCF), where he is also Chief of the Occupational Dermatology Clinic. In his 35 years at UCSF, Dr. Maibach has written and lectured extensively on dermatotoxicology and dermatopharmacology. His current research programs include defining the chemical-biologic faces of irritant dermatitis and the study of percutaneous penetration. Dr. Maibach served on the 1998 ICCVAM Peer Panel that evaluated the LLNA. Dr. Maibach has been on the editorial boards of over 30 scientific journals and is a member of 19 professional societies including the American Academy of Dermatology, San Francisco Dermatological Society, and the International Commission on Occupational Health. He has co-authored over 1500 publications related to dermatology.

Michael Olson, Ph.D., A.T.S.

Dr. Olson received a Ph.D. in Toxicology from the University of Arkansas for Medical Sciences, with dissertation research conducted at the U.S. Food and Drug Administration National Center for Toxicological Research. Following graduate training, he served as NIEHS National Research Service Award Postdoctoral Fellow in the Department of Pharmacology, School of Medicine - University of North Carolina. Currently he is Director, Occupational Toxicology, Corporate Environment Health and Safety for GlaxoSmithKline. Dr. Olson is a Fellow of the Academy of Toxicological Sciences (A.T.S.). His research interests include mechanisms of chemically-induced toxicity; genetic toxicity; xenobiotic metabolism; alternative methods in toxicology; hazard evaluation, risk assessment, and communication. Dr. Olson has authored a number of peer-reviewed manuscripts and book chapters in these areas as well as preparing many occupational health effects reviews for pharmaceutical active ingredients, isolated intermediates, and associated chemicals. He has served as an editorial board member and *ad hoc* referee for numerous toxicology and biosciences journals. In addition, he has worked as a Visiting Scientist, EPA, as well as advisor to EPA Risk Assessment Forum, U.S. National Institutes of Health (NIH) (Toxicology Study Section I), U.S. Air Force, Transportation Research Board, and the National Research Council - National Academy of Sciences (NAS). A member of several biomedical professional societies, Dr. Olson has served in elective and appointed positions in

the Society of Toxicology, including Chairman of the Society of Toxicology (SOT) Occupational Health Specialty Section.

Raymond Pieters, Ph.D.
Dr. Pieters received a Ph.D. at Utrecht University and is currently an Associate Professor at the Institute for Risk Assessment Sciences, and Group Leader for Immunotoxicology at that institution. In 2007, he presented a paper on Development of Strategies to Assess Drug Hypersensitivity at the Congress of the European Societies of Toxicology. He was involved in the development of the Reporter Antigen Popliteal Lymph Node Assay, an assay to assess the immunomodulating potential of chemicals, which enables differentiation between immunosensitizing chemicals (sensitizers), immunostimulating chemicals (irritants), and chemicals that have no apparent immunological effects. He has published over 70 papers on sensitization and other subjects in immunotoxicology in peer-reviewed journals, including a review article, *Murine Models of Drug Hypersensitivity*, in 2005.

Jean Regal, Ph.D.
Dr. Regal received a Ph.D. in Pharmacology from the University of Minnesota. She is currently a Professor in the Department of Pharmacology, Department of Biochemistry and Molecular Biology, University of Minnesota Medical School, Duluth. Her current research is focused on respiratory allergy, especially asthma. She has served on multiple NIH review panels regarding asthma, as an immunotoxicologist in 2000 for an Institute of Medicine Committee on Health Effects Associated with Exposures Experienced during the Persian Gulf War, as well as on the 1998 and 2008 ICCVAM Peer Panel that evaluated the LLNA. In 2007 she served as an ad hoc reviewer for the NTP Board of Scientific Counselors for two nominations: Artificial Butter Flavoring Mixture & O-phthalaldehyde, at NIEHS. She is currently President of the Immunotoxicology Specialty Section of SOT and Associate Editor of the Journal of Immunotoxicology. Dr. Regal has authored over 50 research articles and reviews in peer-reviewed journals.

Jonathan Richmond, B.Sc. (Hons) Med.Sci., MB ChB, FRCSEd, FRMS
Dr. Richmond received a Bachelor of Science in Medical Science with Honors (BSc [Hons] Med.Sci.) and Bachelor of Medicine and Bachelor of Surgery (MB ChB) degrees with Distinction in Medicine and Therapeutics from Edinburgh University. Presently, he is head of the Animals Scientific Procedures Division at the Home Office. He is a Fellow of the Royal College of Surgeons of Edinburgh (FRCSEd) and a former Fellow of the Royal Society of Medicine (FRMS). Other appointments include convener of the U.K. Interdepartmental Group on the 3Rs, convener of the International Standards Organization Technical Corrigendum 194/Working Group 3 (*Biocompatibility of Medical Device*

Materials), and member of related expert working groups. He is a former member of the European Union (E.U.) Committee on Scientific and Technical Progress and past Chairman of the European Commission Technical Expert Working Group on ethical review, and former board member of the U.K. National Centre for the 3Rs. He served as chair of the peer review panel for the reduced LLNA test protocol and prediction model for ESAC in 2007 and has been designated as an ESAC peer reviewer for ECVAM's performance standards for the standard LLNA. He served on the NICEATM-ICCVAM Expert Panel that evaluated Five *In Vitro* Pyrogen Test Methods, and developed performance standards for minor variations on the test method. He has a variety of publications in peer-reviewed journals and national and international meetings, on the principles and practice of surgery, regulation of biomedical research, principles of humane research, bioethics, and public policy.

Peter Theran, V.M.D.

Dr. Theran holds a Doctor of Veterinary Medicine degree from the University of Pennsylvania. He has had many years of experience both as a veterinary internal medicine specialist at the Massachusetts Society for the Prevention of Cruelty to Animals' Angell Memorial Animal Hospital in Boston, and as the director of Boston University Medical Center's Laboratory Animal Science Center. He has served on NIH and NAS committees as an animal welfare member, and is a member of the Board of Directors of the Institute for *In Vitro* Sciences in Gaithersburg, MD, and Chimp Haven in Shreveport, LA. He served on the NICEATM-ICCVAM Expert Panels that evaluated the *In Vitro* Test Methods for Identifying Ocular Corrosives and Severe Irritants, LLNA and *In Vitro* Pyrogen Test Methods. He is a former member of SACATM. He is presently working as an animal welfare consultant.

Stephen Ullrich, Ph.D.

Dr. Ullrich received a Ph.D. in Microbiology from Georgetown University. He is currently the Dallas/Fort Worth Living Legends Professor and Professor of Immunology at the University of Texas, M.D. Anderson Cancer Center, where he is also Associate Director, The Center for Cancer Immunology Research. He is also a member of the Animal Research Strategic Advisory Committee. He has served numerous national review committees and panels, including the 1998 ICCVAM Peer Panel that evaluated the Murine LLNA. Dr. Ullrich has authored over 75 peer-reviewed publications, over 30 invited articles, and he holds four patents in the U.S., E.U., and Australia for a UV-induced Immunosuppressive Substance. He is the past President of the American Society for Photobiology.

Michael Woolhiser, Ph.D.

Dr. Woolhiser received a Ph.D. in Pharmacology and Toxicology from the Medical College of Virginia at Virginia Commonwealth University. He is a specialist in immunotoxicology

and is currently a toxicologist for the Dow Chemical Company, where he serves as a Technical Leader for Immunotoxicology and Polyurethane Business Toxicology Consultant. Dr. Woolhiser is also an Adjunct Assistant Professor at the Center for Integrative Toxicology, Michigan State University. He has served on numerous working groups, including an LLNA Expert Working Group under the European Crop Protection Agency's Toxicology Expert Group, a European Centre for Ecotoxicology and Toxicology of Chemicals LLNA Task Force. He has authored 32 peer-reviewed publications.

Takahiko Yoshida, M.D., Ph.D.

Dr. Yoshida earned his M.D. and a Ph.D. in Medical Science from Tokai University. He is currently Professor in the Department of Health Science at Asahikawa Medical College. Prior to this appointment, he held the posts of Instructor, Assistant Professor, and Associate Professor at the Tokai University School of Medicine. He has also been a Guest Researcher at NIEHS. He has also worked as an occupational physician for major Japanese corporations, including Toyota and Sony. Dr. Yoshida's research interests include occupational health, public health, environmental health, and preventative medicine. He is a member of the International Congress of Occupational Health, the Japanese Society of Hygiene, the Japanese Society of Immunotoxicology, the Japanese Society of Clinical Ecology, and the SOT.

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Appendix E

JaCVAM Statement on the LLNA-DA for Skin Sensitization Testing

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新規試験法提案書

皮膚感作性試験代替法(LLNA-DA 法)

平成 20 年 11 月

国立医薬品食品衛生研究所

新規試験法提案書

平成 20 年 11 月 4 日
No. 2008-02

皮膚感作性試験代替法（LLNA-DA 法）の提案

平成20年8月28日に東京、国立医薬品食品衛生研究所にて開催された新規試験法評価会議（通称：JaCVAM 評価会議）において以下の提案がなされた。

提案内容：皮膚感作性試験代替法(LLNA：Local Lymph Node Assay-DA 法)を定められた方法で適切に利用すれば、化学物質の皮膚感作性を科学的に評価できることを提案する。

この提案書は日本動物実験代替法学会の組織するバリデーション委員会により準備された資料をもとに、日本動物実験代替法学会の組織する評価委員会によりまとめられた文書を用いて JaCVAM 評価会議が OECD ガイダンス文書 No.34 に従って、評価および検討した結果、OECD テストガイドライン No.429（Skin sensitization: Local Lymph Node Assay）に準じて用いることにより、その有用性が確認されたことから作成された。

以上の理由により、行政当局の安全性評価方法として「皮膚感作性試験代替法(LLNA-DA 法)」の使用を提案するものである。

添付資料一覧

1．JaCVAM 評価会議報告書
2．皮膚感作性試験代替法（LLNA－DA 法）の評価報告書（第一次＆第二次評価）
3．LLNA-DA バリデーション研究報告書（第 1 次＆第 2 次実験）
4．ダイセル化学工業株式会社からの追加資料
5．代替試験法申請書 皮膚感作性試験：LLNA－DA 法
6．OECD 429, OECD GUIDELINE FOR THE TESTING CHEMICALS, Skin Sensitization: Local Lymph Node Assay
7．試験法公定化までの道程と JaCVAM の担当

小島 肇

国立医薬品食品衛生研究所
安全性生物試験研究センター
薬理部 新規試験法評価室
室長

井上 達

JACVAM 評価会議 議長
国立医薬品食品衛生研究所
安全性生物試験研究センター
センター長

JaCVAM statement

on the LLNA-DA for skin sensitization testing

At the meeting concerning the above method, held on 28 August 2008 at the National Institute of Health Sciences (NIHS), Tokyo, Japan, the noncommission members of the Japanese Center for the Validation of Alternative Methods (JaCVAM) Regulatory Acceptance Board [1] unanimously endorsed the following statement:

Following the review of the results of the Ministry of Health, Labour and Welfare (MHLW)-funded validation study on the LLNA (Local Lymph Node Assay) -DA coordinated by Japanese Society for Alternative to Animal Experiments (JSAAE), it is concluded that the LLNA-DA can be used for distinguishing between sensitizer and non-sensitizer chemicals within the context of the OECD testing guideline No. 429 on Skin sensitization: LLNA.

The JaCVAM Regulatory Acceptance Board has been regularly kept informed of the progress of the study, and this endorsement is based on an assessment of various documents, including, in particular, the report on the results from the study, and also on the evaluation supported by JSAAE of the study prepared for the JaCVAM ad hoc peer review panel.

Hajime Kojima,
Director,
JaCVAM,
National Centre for Biological Safety and Research (NCBSR)
NIHS,
Tokyo

Tohru Inoue,
Director,
NCBSR,
NIHS,
Tokyo

4 November 2008

1. The JaCVAM Regulatory Acceptance Board was established by the JaCVAM Steering Committee, and is composed of nominees from the industry and academia.

This statement was endorsed by the following members of the JaCVAM Regulatory Acceptance Board:

Mr. Tohru Inoue (NIHS)
Mr. Makoto Hayashi (An-pyo Center*)
Mr. Noriho Tanaka (FDSC*)
Mr. Takemi Yoshida (Showa Univ.)
Ms Masako Mizoguchi (St. Marianna Univ. School of Medicine)
Mr. Fumio Sagami (Eisai Co., Ltd./JPMA*)
Ms Yuko Okamoto (KOSÉ Corporation/JCIA*)
Mr. Hiroshi Onodera (PMDA*)
Mr. Yoshiaki Ikarashi (NIHS)

The following members of the JaCVAM Steering Committee were involved as observers in the consultation process, but not in the endorsement process itself.

Mr. Yasuo Ohno (NIHS)
Mr. Kenichi Nakazawa (NIHS)
Mr. Hiroshi Itagaki (JSAAE)
Mr. Mitsuteru Masuda (JaCVAM)
Mr. Hajime Kojima (JaCVAM)

* An-pyo Cneter: Biosafety Research Center Food, Drugs and Pesticides
FDSC: Food and Drug Safety Center
PMDA: Pharmaceuticals and Medical Devices Agency
JPMA: Japan Pharmaceutical Manufacturers Association
JCIA: Japan Cosmetic Industry Association

Appendix F

Federal Register Notices and Public Comments

F1 *Federal Register* Notices F-3

F2 Public Comments Received in Response to *Federal Register* Notices F-23

F3 Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) Comments: SACATM Meeting on June 18-19, 2008 .....F-107

F4 Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) Comments: SACATM Meeting on June 25-26, 2009 .....F-121

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Appendix F1

Federal Register Notices

72 FR 27815 (May 17, 2007)
The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data F-5

72 FR 52130 (September 12, 2007)
Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments F-8

73 FR 1360 (January 8, 2008)
Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments F-10

73 FR 25754 (May 7, 2008)
Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) F-13

73 FR 29136 (May 20, 2008)
Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments F-15

74 FR 8974 (February 27, 2009)
Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments F-17

74 FR 19562 (April 29, 2009)
Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) F-19

74 FR 26242 (June 1, 2009)
Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for

Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments F-21

DEPARTMENT OF HEALTH AND HUMAN SERVICES

National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); the Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data

AGENCY: National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH).

ACTION: Request for comments, submission of relevant data, and nominations of scientific experts.

SUMMARY: The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) received a nomination from the U.S. Consumer Product Safety Commission (CPSC) to evaluate the validation status of: (1) The murine local lymph node assay (LLNA) as a stand-alone assay for determining potency (including severity) for the purpose of hazard classification; (2) the ''cut-down'' or ''limit dose'' LLNA approach; (3) non-radiolabeled LLNA methods; (4) the use of the LLNA for testing mixtures, aqueous solutions, and metals; and (5) the current applicability domain (i.e., the types of chemicals and substances for which the LLNA has been validated). ICCVAM reviewed the nomination, assigned it a high priority, and proposed that NICEATM and ICCVAM carry out the following activities in its evaluation: (1) Initiate a review of the current literature and available data, including the preparation of a comprehensive background review document, and (2) convene a peer review panel to review the various proposed LLNA uses and procedures for which sufficient data and information are available to adequately assess their validation status. ICCVAM also recommends development of performance standards for the LLNA. At this time, NICEATM requests: (1) Public comments on the appropriateness and relative priority of these activities, (2) nominations of expert scientists to consider as members of a possible peer review panel, and (3) submission of data for the LLNA and/or modified versions of the LLNA.

DATES: Submit comments, data, and nominations by June 15, 2007. Relevant data will also be accepted after this date and considered when feasible.

ADDRESSES: Dr. William S. Stokes, NICEATM Director, NIEHS, P.O. Box 12233, MD EC–17, Research Triangle Park, NC 27709, (fax) 919–541–0947, (e-mail) *niceatm@niehs.nih.gov.* Courier address: NICEATM, 79 T.W. Alexander Drive, Building 4401, Room 3128, Research Triangle Park, NC 27709. Responses can be submitted electronically at the ICCVAM–NICEATM Web site: *http://iccvam.niehs.nih.gov/contact/FR_pubcomment.htm* or by e-mail, mail, or fax.

FOR FURTHER INFORMATION CONTACT: Other correspondence should be

directed to Dr. William S. Stokes (919–541–2384 or *niceatm@niehs.nih.gov*).

SUPPLEMENTARY INFORMATION:

Background

ICCVAM previously evaluated the validation status of the LLNA as a stand-alone alternative method to the Guinea Pig Maximization Test (GPMT) and the Buehler Assay (NIH publication No. 99–4494; available at *http://iccvam.niehs.nih.gov/methods/immunotox/llna.htm*). Based on this evaluation, ICCVAM recommended the LLNA as a valid substitute for the guinea pig methods for most testing situations. The Environmental Protection Agency, Food and Drug Administration, and the CPSC subsequently accepted the method as a valid substitute. The OECD also adopted the LLNA as OECD Test Guideline 429.

In January 2007, the CPSC submitted a nomination to NICEATM (*http://iccvam.niehs.nih.gov/SuppDocs/submission.htm*) requesting that ICCVAM assess the validation status of:

- The LLNA as a stand-alone test for potency determinations (including severity) for the purpose of hazard classification.
- LLNA protocols that do not require the use of radioactive materials.
- The LLNA ''cut-down'' or ''limit dose'' procedure.
- The ability of the LLNA to test mixtures, aqueous solutions, and metals.
- The current applicability domain (i.e., the types of chemicals and substances for which the LLNA has been determined to be useful).

Since 2003, ICCVAM has routinely developed performance standards for test methods; however, they were not developed for the LLNA, which was reviewed in 1999. Accordingly, ICCVAM proposes to now develop performance standards for the LLNA. Performance standards communicate the basis by which new proprietary and nonproprietary test methods have been determined to have sufficient relevance and reliability for specific testing purposes. Performance standards based on test methods accepted by regulatory agencies can be used to evaluate the reliability and relevance of other test methods that are based on similar scientific principles and measure or predict the same biological or toxic effect. On January 24, 2007, ICCVAM unanimously endorsed with a high priority: (1) Developing performance standards for the LLNA and (2) initiating a review of the available data and information associated with the CPSC nominated activities. A determination of which (if any) of the nominated activities will move forward will be made subsequent to this review and after consideration of comments by the public and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM). If a decision is made to proceed with evaluation of these test methods, ICCVAM and NICEATM propose convening a peer review panel to review the usefulness and limitations of each of the LLNA methods listed above. The panel would also formulate conclusions on the adequacy of draft ICCVAM performance standards, any proposed future validation studies, and draft ICCVAM-proposed standardized test method protocols.

Request for Public Comments and Nominations of Scientific Experts

NICEATM requests public comments on the appropriateness and relative priority of the nominated activities. NICEATM also requests the nominations of scientists with relevant knowledge and experience to serve on the panel if a panel meeting occurs. Areas of relevant expertise include, but are not limited to: physiology, pharmacology, immunology, skin sensitization testing in animals, development and use of in vitro methodologies, biostatistics, knowledge about the use of chemical datasets for validation of toxicity studies, and hazard classification of chemicals and products. Each nomination should include the person's name, affiliation, contact information (i.e., mailing address, e-mail address, telephone and fax numbers), curriculum vitae, and a brief summary of relevant experience and qualifications.

Request for Data

NICEATM invites the submission of data from standard LLNA testing (i.e., OECD TG 429) with mixtures, aqueous solutions, and/or metals, as well as corresponding data from human and other animal studies. In addition, NICEATM invites the submission of data supporting the use of (1) the LLNA as a stand-alone test for determining potency (including severity) for the purpose of hazard classification, (2) the LLNA ''cut-down'' or ''limit dose'' procedure, and (3) LLNA protocols that do not require the use of radioactivity. Although data can be accepted at any time, data submitted by June 15, 2007, will be considered during the ICCVAM evaluation process. Submitted data will be used to further evaluate the usefulness and limitations of the LLNA and may be incorporated into future NICEATM and ICCVAM reports and publications as appropriate. The data will also be included in a database to support the investigation of other test methods for assessing skin sensitization.

When submitting chemical and protocol information/test data, please reference this **Federal Register** notice and provide appropriate contact information (name, affiliation, mailing address, phone, fax, e-mail, and sponsoring organization, as applicable).

NICEATM prefers data to be submitted as copies of pages from study notebooks and/or study reports, if available. Raw data and analyses available in electronic format may also be submitted. Each submission for a chemical should preferably include the following information, as appropriate:

- Common and trade name.
- Chemical Abstracts Service Registry Number (CASRN).
- Chemical class.
- Product class.
- Commercial source.
- LLNA protocol used.
- Individual animal responses.
- The extent to which the study complied with national or international Good Laboratory Practice (GLP) guidelines.
- Date and testing organization.
- Sensitization data from other test methods.

Consideration by SACATM

On June 12, 2007, SACATM will meet at the Marriott Bethesda North Hotel and Conference Center in Bethesda, Maryland. The agenda includes consideration of the nominated LLNA activities, priorities, and proposed activities *http://ntp.niehs.nih.gov/go/7441*) and an opportunity for oral public comments. The SACATM meeting was announced in a separate **Federal Register** notice (**Federal Register** Vol. 72, No. 83, pp. 23831–32, May 1, 2007).

Background Information on ICCVAM and NICEATM

ICCVAM is an interagency committee composed of representatives from 15 federal regulatory and research agencies that use or generate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability and promotes the scientific validation and regulatory acceptance of toxicological test methods that more accurately assess the safety and hazards of chemicals and products and that refine, reduce, or replace animal use. The ICCVAM Authorization Act of 2000 (42 U.S.C. 285l–3, available at *http://iccvam.niehs.nih.gov/about/PL106545.htm*) establishes ICCVAM as a permanent interagency committee of the

NIEHS under NICEATM. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of federal agencies. Additional information about ICCVAM and NICEATM is available on the following Web site: *http://iccvam.niehs.nih.gov*.

Dated: May 8, 2007.

David A. Schwartz,

Director, National Institute of Environmental Health Sciences and National Toxicology Program.

[FR Doc. E7–9544 Filed 5–16–07; 8:45 am]

BILLING CODE 4140–01–P

DEPARTMENT OF HEALTH AND HUMAN SERVICES

National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments

AGENCY: National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH).

ACTION: Request for comments.

SUMMARY: The Murine Local Lymph Node Assay (LLNA) is the first alternative test method evaluated and recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). It was subsequently accepted by regulatory authorities to determine the allergic contact dermatitis potential of chemicals and products. In January 2007, the U.S. Consumer Product Safety Commission (CSPC) submitted a nomination requesting that NICEATM and ICCVAM assess the validation status of (1) The LLNA as a stand-alone assay for potency determination for hazard classification purposes; (2) modified LLNA protocols; (3) the LLNA limit test; (4) the use of LLNA to test mixtures, aqueous solutions, and metals; and (5) the applicability domain for LLNA. In order to facilitate the review of the modified LLNA protocols, ICCVAM proposed developing performance standards for the LLNA. In May 2007, a **Federal Register** notice was published (Vol. 72, No. 95, pages 27815–27817, May 17, 2007) requesting comments and data relevant to these nominated activities. In June 2007, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) endorsed the nominated activities as high priorities for ICCVAM. In response to SACATM comments, along with those provided by the public in response to the previous **Federal Register** notice, ICCVAM also endorsed these activities as high priorities. ICCVAM subsequently prepared draft performance standards for the LLNA and now requests public comments on this draft document, which is available on the NICEATM/ICCVAM Web site at: (*http://iccvam.niehs.nih.gov/methods/immunotox/immunotox.htm*) or by contacting NICEATM (see **FOR FURTHER INFORMATION CONTACT** below).

DATES: Submit comments on or before October 29, 2007.

ADDRESSES: Dr. William S. Stokes, NICEATM Director, NIEHS, P.O. Box 12233, MD EC–17, Research Triangle Park, NC 27709, (fax) 919–541–0947, (e-mail) *niceatm@niehs.nih.gov*. Courier address: NICEATM, 79 T.W. Alexander Drive, Building 4401, Room 3128, Research Triangle Park, NC 27709. Responses can be submitted electronically at the ICCVAM–NICEATM Web site: *http://iccvam.niehs.nih.gov/contact/FR_pubcomment.htm* or by e-mail, mail, or fax.

FOR FURTHER INFORMATION CONTACT: Other correspondence should be directed to Dr. William S. Stokes (919–541–2384 or *niceatm@niehs.nih.gov*).

SUPPLEMENTARY INFORMATION:

Background

The LLNA is an alternative test method used for skin sensitization testing that reduces the number of animals needed, reduces the time required for testing, and can substantially reduce or avoid pain and distress associated with traditional guinea pig testing methods. The LLNA was the first alternative test method evaluated and recommended by ICCVAM and based on the recommendations of ICCVAM and an independent scientific peer review panel, the LLNA has been accepted by U.S. and international regulatory authorities as an alternative to the guinea pig maximization test and Buehler test for assessing allergic contact dermatitis (EPA 2003; ISO 2002; OECD 2002). Since 2003, ICCVAM has routinely developed performance standards for test methods; however, because the concept of performance standards was not developed by ICCVAM until 2003, they were not developed during the ICCVAM evaluation of the LLNA in 1998 (NIH Publication No. 99–4494, available: (*http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf*).

In January 2007, CSPC submitted a nomination requesting that NICEATM and ICCVAM assess the validation status of (1) The LLNA as a stand-alone assay for potency determination for classification purposes; (2) modified LLNA protocols; (3) the LLNA limit test; (4) the use of LLNA to test mixtures, aqueous solutions, and metals; and (5) the applicability domain for LLNA. ICCVAM endorsed the nomination and also decided to develop performance standards to facilitate evaluation of modified LLNA protocols to the traditional LLNA. In May 2007, a **Federal Register** notice was published requesting comments and data relevant to these activities (Vol. 72, No. 95, pages 27815–27817, May 17, 2007; available,

http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf). In June 2007, SACATM endorsed these activities as high priorities for ICCVAM. In response to SACATM comments, along with those provided by the public in response to the previous **Federal Register** notice, ICCVAM endorsed these activities, including the development of performance standards, as high priorities. ICCVAM subsequently prepared draft performance standards for the LLNA, which are available on the NICEATM/ICCVAM Web site at: (*http://iccvam.niehs.nih.gov/methods/immunotox/immunotox.htm*).

These draft test method performance standards are proposed to evaluate the performance of LLNA test methods that incorporate specific modifications to the measurement of lymphocyte proliferation in the traditional LLNA. These modifications focus specifically on incorporating non-radioactive procedures to evaluate lymphocyte proliferation in the draining auricular lymph nodes rather than incorporation of radioactivity (i.e., ^{3}H-thymidine), which is used in the traditional LLNA.

Public comments received in response to the draft LLNA performance standards will be considered by ICCVAM during development of a revised draft version of this document. A public meeting is planned for early 2008 where an international, independent, peer review panel will evaluate the revised draft LLNA performance standards and review the other nominated LLNA related activities. Following this meeting, the recommendations of the peer review panel will be made available for public and SACATM comment. ICCVAM will consider the panel report and public and SACATM comments in preparing final LLNA performance standards.

Request for Public Comments

NICEATM invites the submission of written comments on the draft LLNA performance standards. When submitting written comments, please refer to this **Federal Register** notice and include appropriate contact information (name, affiliation, mailing address, phone, fax, e-mail, and sponsoring organization, if applicable). All comments received by the deadline listed above will be placed on the NICEATM/ICCVAM Web site (*http://ntp-apps.niehs.nih.gov/iccvampb/searchPubCom.cfm*) and made available to the peer review panel and ICCVAM.

Background Information on ICCVAM and NICEATM

ICCVAM is an interagency committee composed of representatives from 15 federal regulatory and research agencies that use or generate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability and promotes the scientific validation and regulatory acceptance of toxicological test methods that more accurately assess the safety and hazards of chemicals and products and that refine, reduce, or replace animal use. The ICCVAM Authorization Act of 2000 (42 U.S.C. 285*l*–3, available at *http://iccvam.niehs.nih.gov/about/PL106545.htm*) establishes ICCVAM as a permanent interagency committee of the NIEHS under NICEATM. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of federal agencies. Additional information about ICCVAM and NICEATM is available on the following Web site: *http://iccvam.niehs.nih.gov*.

Dated: September 5, 2007.

Samuel H. Wilson,

Acting Director, National Institute of Environmental Health Sciences and National Toxicology Program.

[FR Doc. E7–18011 Filed 9–11–07; 8:45 am]

BILLING CODE 4140–01–P

DEPARTMENT OF HEALTH AND HUMAN SERVICES

National Institutes of Health

National Toxicology Program (NTP); NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments

AGENCY: National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH).

ACTION: Meeting announcement and request for comments.

SUMMARY: NICEATM in collaboration with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) announces an independent scientific peer review panel meeting to evaluate modifications and new applications for the Murine Local Lymph Node Assay (LLNA). The LLNA is an alternative test method that can be used to determine the allergic contact dermatitis potential of chemicals and products. The panel will review the following:

- The validation status of three modified LLNA test method protocols that use non-radioactive probe chemicals.
- The validation status of a LLNA limit dose procedure.
- The use of the LLNA to test mixtures, aqueous solutions, and metals (applicability domain for the LLNA).
- The use of the LLNA to determine potency (potential for causing allergic contact dermatitis).
- Revised draft recommended performance standards for the LLNA.

At this meeting, the panel will peer review the draft background review documents and revised draft LLNA performance standards for each topic and evaluate the extent that established validation and acceptance criteria have been appropriately addressed. The panel will also comment on the extent

that the review documents support draft ICCVAM recommendations on proposed test method protocols, proposed uses of the LLNA, and the revised draft LLNA performance standards.

NICEATM invites public comments on the draft background review documents, draft ICCVAM test recommendations, draft test method protocols, and revised draft LLNA performance standards. All documents will be available on the NICEATM–ICCVAM Web site at *http://iccvam.niehs.nih.gov/methods/immunotox/immunotox.htm* by January 8, 2008.

DATES: The meeting is scheduled for March 4–6, 2008, from 8:30 a.m. to 5 p.m. each day. The meeting is open to the public free of charge, with attendance limited only by the space available. In order to facilitate planning for this meeting, persons wishing to attend are asked to register by February 20, 2008, via the NICEATM–ICCVAM Web site (*http://iccvam.niehs.nih.gov/contact/reg_LLNAPanel.htm*). The deadline for written comments is February 22, 2008.

ADDRESSES: The meeting will be held at the U.S. Consumer Product Safety Commission (CPSC) Headquarters, Bethesda Towers Bldg., 4330 East West Highway, Bethesda, MD.

FOR FURTHER INFORMATION CONTACT: Comments may also be submitted via the NICEATM–ICCVAM Web site at *http://iccvam.niehs.nih.gov/contact/FR_pubcomment.htm*. Comments or other correspondence can be sent to Dr. William S. Stokes, NICEATM Director, NIEHS, P.O. Box 12233, MD EC–17, Research Triangle Park, NC, 27709, (phone) 919–541–2384, (fax) 919–541–0947, (e-mail) *niceatm@niehs.nih.gov*. Courier address: NICEATM, 79 T.W. Alexander Drive, Building 4401, Room 3128, Research Triangle Park, NC 27709.

SUPPLEMENTARY INFORMATION:

Background

The LLNA is a reduction and refinement alternative test method for skin sensitization testing because it reduces the number of animals needed and can substantially reduce or avoid pain and distress compared to traditional guinea pig testing methods for sensitization. The LLNA was the first alternative test method evaluated and recommended by ICCVAM (NIH Publication No. 99–4494, available at: *http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf*). Based on the recommendations of ICCVAM and an independent scientific peer review panel, U.S. and international regulatory authorities have accepted the LLNA as an alternative to the guinea pig maximization test and Buehler test for assessing allergic contact dermatitis (ISO 2002; OECD 2002; EPA 2003). This review will evaluate the potential for broader use of the LLNA for regulatory testing of chemicals and products for allergic contact dermatitis potential, enabling further reduction and refinement (less pain and suffering) of animal use for this purpose. In January 2007, the CPSC submitted a nomination requesting that NICEATM and ICCVAM assess the validation status of (1) the LLNA as a stand-alone assay for potency determination for hazard classification purposes; (2) modified LLNA protocols; (3) the LLNA limit test; (4) the use of the LLNA to test mixtures, aqueous solutions, and metals; and (5) the applicability domain for the LLNA. In June 2007, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) endorsed these activities as high priorities for ICCVAM. NICEATM on behalf of ICCVAM also sought input from the public on these activities (**Federal Register**: Vol. 72, No. 95, pages 27815–27817, May 17, 2007). After considering these inputs, ICCVAM endorsed these activities as high priorities. ICCVAM is also developing performance standards to facilitate evaluation of modified LLNA protocols compared to the traditional LLNA. Although ICCVAM has routinely developed performance standards for test methods since 2003, they were not developed as part of the ICCVAM evaluation of the LLNA in 1998. These draft performance standards for the LLNA were made public and comments were requested via the **Federal Register** (Vol. 72, No. 176, pages 52130–52131, Sept. 12, 2007). The May 2007 **Federal Register** notice requested data from studies using the LLNA or modified versions of the LLNA.

Drawing on the submitted data and literature sources, ICCVAM and NICEATM drafted background review documents for each of the modifications and new applications of the LLNA. ICCVAM has also developed draft test method recommendations regarding the proposed usefulness, limitations, and validation status of these test methods. ICCVAM will convene an independent scientific panel to peer review the draft background review documents for the test methods and determine whether the data and analyses in the draft documents support the draft ICCVAM test method recommendations. The panel will also be asked to comment on the adequacy of the revised draft performance standards, proposed future studies, draft standardized test method protocols, and recommended reference substances. NICEATM will ask the panel to consider all available information, including the scientific studies cited in the draft review documents, public comments, and any new information identified during the peer review, for developing their conclusions and recommendations.

Peer Review Panel Meeting

The purpose of this meeting is to conduct a scientific peer review of the revised draft performance standards and an evaluation of modifications and new applications for the LLNA. The LLNA is an alternative test method that can be used to determine the allergic contact dermatitis potential of chemicals and products. The panel will review the following:

- The LLNA as a stand-alone assay for potency determination for hazard classification purposes
- Modified LLNA protocols
- The LLNA limit test
- The use of the LLNA to test mixtures, aqueous solutions, and metals (applicability domain for the LLNA)
- The use of the LLNA to determine potency (potential for causing allergic contact dermatitis).

The panel will consider the draft background review documents for each of these methods and evaluate the extent that established validation and acceptance criteria are appropriately addressed for each test method (as described in the ICCVAM document, *Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods,* NIH Publication No. 97–981, available at *http://iccvam.niehs.nih.gov/docs/about_docs/validate.pdf*). The panel will then comment on the extent to which the draft ICCVAM recommendations are supported by the information provided in the background review document for each topic. It is anticipated that the panel will address the topics in the following order:

1. The LLNA limit test.
2. The applicability domain of the LLNA including its suitability for mixtures, aqueous solutions, and metals.
3. The LLNA as a stand-alone assay for potency determination for hazard classification.
4. The revised draft performance standards for the LLNA.
5. The modified LLNA test method protocols using non-radioactive materials.

Additional information about the meeting, including a roster of the panel members and the draft agenda, will be made available two weeks prior to the meeting on the NICEATM-ICCVAM Web site (*http://iccvam.niehs.nih.gov*). This information will also be available after that date by contacting NICEATM (see **FOR FURTHER INFORMATION CONTACT** above).

Attendance and Registration

This public meeting will take place March 4–6, 2008, at the CPSC Headquarters, Bethesda Towers Bldg., 4330 East West Highway, Bethesda, MD (an area map, driving directions, and CPSC contact information are available at *http://www.cpsc.gov/about/contact.html*). The meeting will begin at 8:30 a.m. and is scheduled to conclude at approximately 5 p.m. each day, although adjournment on March 6 may occur earlier or later depending upon the time needed for the expert panel to complete its work. It is also possible that the panel may conclude its deliberations on March 5 and not need to meet on March 6. Persons needing special assistance in order to attend, such as sign language interpretation or other reasonable accommodation, should contact 919–541–2475 (voice), 919–541–4644 TTY (text telephone, through the Federal TTY Relay System at 800–877–8339), or e-mail *niehsoeeo@niehs.nih.gov*. Requests should be made at least seven days in advance of the event.

Availability of the Draft Background Review Documents and Draft ICCVAM Recommendations

NICEATM prepared draft background review documents on each of these modifications or applications of the LLNA that describe the current validation status of the modified test methods and applications and contain all of the data and analyses supporting this proposed validation status. The draft background review documents, draft ICCVAM test method recommendations, draft test method protocols, and revised draft test method performance standards are available from the NICEATM–ICCVAM Web site (*http://iccvam.niehs.nih.gov/methods/immunotox/immunotox.htm*) or by contacting NICEATM (see **FOR FURTHER INFORMATION CONTACT** above).

Request for Public Comments

NICEATM invites the submission of written comments on the draft background review documents, draft ICCVAM test method recommendations, draft test method protocols, and revised draft test method performance standards. Written comments should be submitted preferably electronically via the NICEATM–ICCVAM Web site or by e-mail (*niceatm@niehs.nih.gov*); the deadline for submission of written comments is February 22, 2008. When submitting written comments, please refer to this **Federal Register** notice and include appropriate contact information (name, affiliation, mailing address, phone, fax, e-mail, and sponsoring organization, if applicable). Written comments may also be sent by mail, fax, or e-mail to Dr. William Stokes (see **FOR FURTHER INFORMATION CONTACT** above). All comments received will be placed on the NICEATM–ICCVAM Web site (*http://iccvam.niehs.nih.gov*) and identified by the individual's name and affiliation or sponsoring organization (if applicable). Comments will also be sent to the panel and ICCVAM agency representatives and made available at the meeting.

This meeting is open to the public, and time will be provided for the presentation of oral comments by the public at designated times during the peer review. Members of the public who wish to present oral statements at the meeting should contact NICEATM (see **FOR FURTHER INFORMATION CONTACT** above) no later than February 20, 2008, and provide contact information (name, affiliation, mailing address, phone, fax, e-mail, and sponsoring organization, if applicable). Up to seven minutes will be allotted per speaker, one speaker per organization. Persons registering to make comments are asked to provide NICEATM a written copy of their statement by February 27, 2008, so that copies can be distributed to the panel prior to the meeting. If this is not possible, please bring 40 copies of your comments to the meeting for distribution and to supplement the record. Written statements can supplement and expand the oral presentation.

Summary minutes and the panel's final report will be available following the meeting on the NICEATM–ICCVAM Web site (*http://iccvam.niehs.nih.gov*). ICCVAM will consider the panel's conclusions and recommendations and any public comments received when finalizing their test method recommendations and performance standards for these methods.

Background Information on ICCVAM and NICEATM

ICCVAM is an interagency committee composed of representatives from 15 Federal regulatory and research agencies that use or generate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability, and promotes the scientific validation and regulatory acceptance of toxicological test methods that more accurately assess the safety and hazards of chemicals and products and that refine, reduce, or replace animal use. The ICCVAM Authorization Act of 2000 (42 U.S.C. 285l–3, available at *http://iccvam.niehs.nih.gov/docs/about_docs/PL106545.pdf*) establishes ICCVAM as a permanent interagency committee of the NIEHS under NICEATM. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of Federal agencies. Additional information about ICCVAM and NICEATM is available on the NICEATM–ICCVAM Web site at *http://iccvam.niehs.nih.gov*.

References

EPA. 2003. EPA OPPTS 870.2600 Test Guideline—Skin Sensitization. Available: *http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Drafts/870–2600.pdf*.

ISO. 2002. ISO 10993–10 Biological evaluation of medical devices—Part 10: Tests for irritation and delayed-type hypersensitivity. Geneva: International Organization for Standardization.

OECD. 2002. OECD Guideline for the Testing of Chemicals—Test Guideline 429: Skin Sensitization: Local Lymph Node Assay (adopted 24 April 2002). Paris: Organisation for Economic Co-operation and Development.

Dated: December 19, 2007.

Samuel H. Wilson,

Acting Director, National Institute of Environmental Health Sciences and National Toxicology Program.

[FR Doc. E7–25553 Filed 1–7–08; 2:42 pm]

BILLING CODE 4140–01–P

DEPARTMENT OF HEALTH AND HUMAN SERVICES

National Toxicology Program (NTP); Office of Liaison, Policy and Review; Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

AGENCY: National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH).

ACTION: *COM057*Meeting announcement and request for comment.

SUMMARY: Pursuant to section 10(a) of the Federal Advisory Committee Act, as amended (5 U.S.C. Appendix 2), notice is hereby given of a meeting of SACATM on June 18–19, 2008, at the

Radisson Hotel Research Triangle Park, 150 Park Drive, Research Triangle Park, NC 27709. The meeting is scheduled from 8:30 a.m. to 5:30 p.m. on June 18 and 8:30 a.m. until adjournment on June 19. The meeting is open to the public with attendance limited only by the space available. SACATM advises the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and the Director of the NIEHS and NTP regarding statutorily mandated duties of ICCVAM and activities of NICEATM.

DATES: The SACATM meeting will be held on June 18 and 19, 2008. All individuals who plan to attend are encouraged to register online at the NTP Web site (*http://ntp.niehs.nih.gov/go/7441*) by June 10, 2008. In order to facilitate planning, persons wishing to make an oral presentation are asked to notify Dr. Lori White, NTP Executive Secretary, via online registration, phone, or email by June 10, 2008 (see **ADDRESSES** below). Written comments should also be received by June 10 to enable review by SACATM and NIEHS/NTP staff before the meeting.

ADDRESSES: The SACATM meeting will be held at the Radisson Hotel Research Triangle Park, 150 Park Drive, Research Triangle Park, NC 27709 [hotel: (919) 549–8631]. Public comments and other correspondence should be directed to Dr. Lori White (NTP Office of Liaison, Policy and Review, NIEHS, P.O. Box 12233, MD A3–01, Research Triangle Park, NC 27709; telephone: 919–541–9834 or e-mail: *whiteld@niehs.nih.gov*). Courier address: NIEHS, 111 T.W. Alexander Drive, Room A326, Research Triangle Park, NC 27709. Persons needing interpreting services in order to attend should contact 301–402–8180 (voice) or 301–435–1908 (TTY). Requests should be made at least 7 days in advance of the meeting.

SUPPLEMENTARY INFORMATION:

Preliminary Agenda Topics and Availability of Meeting Materials

Preliminary agenda topics include:

- NICEATM–ICCVAM Update;
- Overview of NICEATM–ICCVAM 5–Year Plan;
- NRC Report: Toxicity Testing in the 21st Century;
- Presentations from Federal Agencies on Research, Development, Translation, and Validation Activities Relevant to the NICEATM–ICCVAM Five-Year Plan;
- Report on the ICCVAM–NICEATM Independent Scientific Peer Review Meeting: Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA), a Test Method for Assessing the Contact Dermatitis Potential of Chemicals and Products;
- Report on the ICCVAM–NICEATM–ECVAM–JACVAM Scientific Workshop on Acute Chemical Safety Testing: Advancing In Vitro Approaches and Humane Endpoints for Systemic Toxicity Evaluations;
- Nominations to ICCVAM: NTP Rodent Bioassay for Carcinogenicity;
- Proposal for International Cooperation on Alternative Test Methods;
- Update from the Japanese Center for the Validation of Alternative Methods;
- Update from the European Center for the Evaluation of Alternative Methods,

A copy of the preliminary agenda, committee roster, and additional information, when available will be posted on the NTP Web site (*http://ntp.niehs.nih.gov/go/7441*) or available upon request (see **ADDRESSES** above). Following the SACATM meeting, summary minutes will be prepared and available on the NTP website or upon request.

Request for Comments

Both written and oral public input on the agenda topics is invited. Written comments received in response to this notice will be posted on the NTP Web site. Persons submitting written comments should include their name, affiliation (if applicable), and sponsoring organization (if any) with the document. Time is allotted during the meeting for presentation of oral comments and each organization is allowed one time slot per public comment period. At least 7 minutes will be allotted for each speaker, and if time permits, may be extended up to 10 minutes at the discretion of the chair. Registration for oral comments will also be available on-site, although time allowed for presentation by on-site registrants may be less than for pre-registered speakers and will be determined by the number of persons who register at the meeting.

Persons registering to make oral comments are asked to do so through the online registration form (*http://ntp.niehs.nih.gov/go/7441*) and to send a copy of their statement to Dr. White (see **ADDRESSES** above) by June 10 to enable review by SACATM, NICEATM–ICCVAM, and NIEHS/NTP staff prior to the meeting. Written statements can supplement and may expand the oral presentation. If registering on-site and reading from written text, please bring 40 copies of the statement for distribution and to supplement the record.

Background Information on ICCVAM, NICEATM, and SACATM

ICCVAM is an interagency committee composed of representatives from 15 Federal regulatory and research agencies that use, generate, or disseminate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability and promotes the development, scientific validation, regulatory acceptance, implementation, and national and international harmonization of new, revised, and alternative toxicological test methods that more accurately assess the safety and hazards of chemicals and products and that refine, reduce, and replace animal use. The ICCVAM Authorization Act of 2000 [42 U.S.C. 285l–3] established ICCVAM as a permanent interagency committee of the NIEHS under NICEATM. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of U.S. Federal agencies. Additional information about ICCVAM and NICEATM can be found on their Web site (*http://iccvam.niehs.nih.gov*).

SACATM was established in response to the ICCVAM Authorization Act [Section 285l–3(d)] and is composed of scientists from the public and private sectors. SACATM advises ICCVAM, NICEATM, and the Director of the NIEHS and NTP regarding statutorily mandated duties of ICCVAM and activities of NICEATM. SACATM provides advice on priorities and activities related to the development, validation, scientific review, regulatory acceptance, implementation, and national and international harmonization of new, revised, and alternative toxicological test methods. Additional information about SACATM, including the charter, roster, and records of past meetings, can be found at *http://ntp.niehs.nih.gov/go/167.*

Dated: April 28, 2008.

Samuel H. Wilson,

Acting Director, National Institute of Environmental Health Sciences and National Toxicology Program.

[FR Doc. E8–10010 Filed 5–6–08; 8:45 am]

BILLING CODE 4140–01–P

DEPARTMENT OF HEALTH AND HUMAN SERVICES

National Toxicology Program (NTP); NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

AGENCY: National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH).

ACTION: Request for comments.

SUMMARY: NICEATM, in collaboration with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), convened an independent international scientific peer review panel on March 4–6, 2008 to evaluate new versions and applications of the LLNA for assessing the allergic contact dermatitis potential of chemicals and products. The peer review panel (''the Panel'') report from this meeting is now available. The report contains (1) the Panel's evaluation of the validation status of the methods and (2) the Panel's comments and conclusions on draft ICCVAM test method recommendations. NICEATM invites public comment on the Panel's report. The report is available on the NICEATM–ICCVAM Web site at *http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm* or by contacting NICEATM at the address given below.

DATES: Written comments on the Panel report should be received by July 7, 2008.

ADDRESSES: Comments should be submitted preferably electronically via the NICEATM–ICCVAM Web site at *http://iccvam.niehs.nih.gov/contact/FR_pubcomment.htm*. Comments can also be submitted by e-mail to *niceatm@niehs.nih.gov*. Written comments can be sent by mail or fax to Dr. William S. Stokes, Director, NICEATM, NIH/NIEHS, P.O. Box 12233, MD EC–17, Research Triangle Park, NC 27709, (phone) 919–541–2384, (fax) 919–541–0947. Courier address: NICEATM, 79 T.W. Alexander Drive, Building 4401, Room 3128, Research Triangle Park, NC 27709.

FOR FURTHER INFORMATION CONTACT: Dr. William S. Stokes, Director, NICEATM (919–541–2384 or *niceatm@niehs.nih.gov*).

SUPPLEMENTARY INFORMATION:

Background

In January 2007, the Consumer Product Safety Commission submitted a nomination to NICEATM and ICCVAM to assess the validation status of (1) The use of the LLNA to determine potency for hazard classification purposes; (2) LLNA protocols using non-radioactive procedures; (3) the LLNA limit dose procedure; and (4) the use of the LLNA to test mixtures, aqueous solutions, and metals (*i.e.*, an updated assessment of the applicability domain of the LLNA). In June 2007, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) endorsed these activities as high priorities for ICCVAM. NICEATM, on behalf of ICCVAM, also sought input from the public on these activities and requested data from studies using the LLNA or modified versions of the LLNA (**Federal Register** Vol. 72, No. 95, pages 27815–27817, May 17, 2007). After considering all comments received, ICCVAM endorsed carrying out these activities as high priorities. ICCVAM also developed draft LLNA performance standards to facilitate evaluation of modified LLNA protocols that are functionally and mechanistically similar to the traditional LLNA. These draft LLNA performance standards were made public and comments were requested via the **Federal Register** (Vol. 72, No. 176, pages 52130–52131, Sept. 12, 2007).

ICCVAM and NICEATM prepared draft background review documents (BRDs) that provided comprehensive reviews of available data and relevant information for each of the modifications and new applications of the LLNA. ICCVAM also developed draft test method recommendations regarding the proposed usefulness and limitations, standardized protocols, and future studies. Both the draft BRDs and draft recommendations were made available for public comment, and a public peer review meeting was announced in the **Federal Register** (Vol. 73, No. 5, pages 1360–1362, Jan. 8, 2008).

The Panel met in public session on March 4–6, 2008. The Panel reviewed the draft ICCVAM BRDs for completeness, errors, and omissions of any existing relevant data or information. The Panel evaluated the information in the BRDs to determine the extent to which each of the applicable criteria for validation and acceptance of toxicological test methods (ICCVAM, 2003) had been appropriately addressed. The Panel then considered the ICCVAM draft test method

recommendations (*i.e.*, proposed test method uses, proposed recommended standardized protocol, proposed test method performance standards, and proposed additional studies) and commented on whether the recommendations were supported by the information provided in the draft BRDs.

The Panel's conclusions and recommendations are detailed in the *Peer Review Panel Final Report: Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products* (available at *http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm*). The draft BRDs, draft ICCVAM test method recommendations, and the draft LLNA Performance Standards are available at *http://iccvam.niehs.nih.gov/methods/immunotox/immunotox.htm*.

Request for Comments

NICEATM invites the submission of written comments on the Panel's report. When submitting written comments, please refer to this **Federal Register** notice and include appropriate contact information (name, affiliation, mailing address, phone, fax, e-mail, and sponsoring organization, if applicable). All comments received will be made publicly available on the NICEATM–ICCVAM Web site at *http://ntp-apps.niehs.nih.gov/iccvampb/searchPubCom.cfm*. In addition, there will be an opportunity for oral public comments on the Panel's report during an upcoming meeting of SACATM scheduled for June 18–19, 2008. Information concerning the SACATM meeting will be published in a separate **Federal Register** notice and available on the SACATM Web site at *http://ntp.niehs.nih.gov/go/7441*.

ICCVAM will consider the Panel report along with SACATM and public comments when finalizing test method recommendations. An ICCVAM test method evaluation report, which will include the final ICCVAM recommendations, will be forwarded to relevant Federal agencies for their consideration. The evaluation report will also be available to the public on the NICEATM–ICCVAM Web site and by request from NICEATM (see **ADDRESSES** above).

Background Information on ICCVAM, NICEATM, and SACATM

ICCVAM is an interagency committee composed of representatives from 15 Federal regulatory and research agencies that use, generate, or disseminate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability and promotes scientific validation, regulatory acceptance, and national and international harmonization of toxicological test methods that more accurately assess safety and hazards of chemicals and products and that refine, reduce, and replace animal use. The ICCVAM Authorization Act of 2000 (42 U.S.C. 285*l*-3, available at *http://iccvam.niehs.nih.gov/docs/about_docs/PL106545.pdf*) established ICCVAM as a permanent interagency committee of the NIEHS under NICEATM. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of Federal agencies. Additional information about ICCVAM and NICEATM can be found at the NICEATM–ICCVAM Web site (*http://iccvam.niehs.nih.gov*).

Additional information about SACATM, including the charter, roster, and records of past meetings, can be found at *http://ntp.niehs.nih.gov/go/167*.

References

ICCVAM, 2003, ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods. NIH Publication No. 03–4508. Research Triangle Park, NC: NIEHS. Available at: *http://iccvam.niehs.nih.gov*.

Dated: May 8, 2008.

Samuel H. Wilson,

Acting Director, National Institute of Environmental Health Sciences and National Toxicology Program.

[FR Doc. E8–11195 Filed 5–19–08; 8:45 am]

BILLING CODE 4140–01–P

DEPARTMENT OF HEALTH AND HUMAN SERVICES

National Institutes of Health

National Toxicology Program (NTP); NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments

AGENCY: National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH).

ACTION: Meeting announcement and request for comments.

SUMMARY: NICEATM, in collaboration with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), announces a second meeting of an independent scientific peer review panel (hereafter, Panel) to evaluate three non-radioactive modified versions and new applications for the Murine Local Lymph Node Assay (LLNA). The LLNA is an alternative test method that can be used to determine the allergic contact dermatitis potential of chemicals and products.

The Panel will consider additional data and information for the three non-radioactive modified versions and new applications of the LLNA obtained by NICEATM subsequent to the original Panel meeting in March 2008. Based on this new information, the Panel will review the following:

- The validation status of three modified LLNA test methods.
- The proposed applicability domain of the LLNA.

The Panel will peer review revised draft BRDs for each topic and evaluate the extent that established validation and acceptance criteria have been appropriately addressed. The Panel also will be asked to comment on the extent to which draft ICCVAM test method recommendations are supported by the data analyses provided in the BRDs.

NICEATM invites public comments on the draft BRDs and draft ICCVAM test recommendations. All documents will be available on the NICEATM–ICCVAM Web site at *http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm* by March 3, 2009.

DATES: The meeting is scheduled for April 28–29, 2009 from 8:30 a.m. to 5 p.m. each day. The deadline for registration and submission of written comments is April 14, 2009.

ADDRESSES: The meeting will be held at the Natcher Conference Center, National Institutes of Health, 45 Center Drive, Bethesda, MD 20892. Persons needing special assistance in order to attend, such as sign language interpretation or other reasonable accommodation, should contact 301–402–8180 (voice) or 301–435–1908 TTY (text telephone). Requests should be made at least seven business days in advance of the event.

FOR FURTHER INFORMATION CONTACT: Dr. William S. Stokes, Director, NICEATM, NIEHS, P.O. Box 12233, Mail Stop: K2–16, Research Triangle Park, NC 27709; (telephone) 919–541–2384; (fax) 919–541–0947; (e-mail) *niceatm@niehs.nih.gov*. Courier address: NICEATM, NIEHS, 530 Davis Drive, Room 2035, Mail Stop: K2–16, Durham, NC 27713.

SUPPLEMENTARY INFORMATION:

Background

In January 2007, the U.S. Consumer Product Safety Commission (CPSC) submitted a nomination to NICEATM (*http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf*) requesting that ICCVAM assess the validation status of (1) the LLNA limit dose procedure; (2) three modified LLNA test method protocols that use non-radioactive probe chemicals; (3) the use of the LLNA to test mixtures, aqueous solutions, and metals (applicability domain for the LLNA); and (4) the use of the LLNA to determine potency (potential for causing allergic contact dermatitis). NICEATM compiled draft BRDs that provided comprehensive reviews of the available data and relevant information, which were used as the basis for draft ICCVAM test method recommendations. These documents were released to the Panel and the public for review and comment in January 2008 (73FR1360).

In March 2008, NICEATM and ICCVAM convened the public Panel meeting during which the Panel concluded that more information and data were required for the three modified LLNA test methods before recommendations could be made regarding their use for regulatory safety testing. Similarly, the Panel concluded that more data would be needed before a recommendation on the usefulness and limitations on the current applicability domain of the traditional LLNA could be made. The Panel's conclusions are detailed in a report, which was made available in May 2008 (73FR29136), and includes consideration of public comments made prior to and during their deliberations.

Subsequent to the Panel meeting, NICEATM received additional LLNA data for pesticide formulations and other products, as well as new data for the three modified LLNA test methods. Using the additional information, NICEATM revised the BRDs for each of these modified test methods and new applications of the LLNA. The revised draft BRDs provide all of the data and analyses supporting the scientific validity of the modified test methods and proposed applications. ICCVAM prepared revised draft test method recommendations regarding the proposed usefulness and limitations, standardized protocol, and future studies. NICEATM will reconvene the Panel to consider the additional information and revised recommendations.

Peer Review Panel Meeting

This meeting will take place April 28–29, 2009, at the Natcher Conference Center, National Institutes of Health, 45 Center Drive, Bethesda, Maryland, 20892. It will begin at 8:30 a.m. and is scheduled to conclude at approximately 5 p.m. on each day. The meeting is open to the public at no charge, with attendance limited only by the space available. The Panel will consider the revised draft BRDs for each of these modified versions and new applications of the LLNA and evaluate the extent that established validation and acceptance criteria are appropriately addressed for each test method and application (as described in the ICCVAM document, *Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods*, NIH Publication No. 97–3981, available at *http://iccvam.niehs.nih.gov/docs/about_docs/validate.pdf*). The Panel will then comment on the extent to which each of the revised draft ICCVAM test method recommendations is supported by the information provided in the corresponding revised draft BRDs. The Panel is expected first to review the three modified LLNA test methods, and then review the use of the LLNA for testing pesticide formulations and other products.

Additional information about the Panel meeting, including a roster of the Panel members and the draft agenda, will be made available two weeks prior to the meeting on the NICEATM–ICCVAM Web site (*http://iccvam.niehs.nih.gov*). This information will also be available after that date by

contacting NICEATM (see **FOR FURTHER INFORMATION CONTACT** above).

Attendance and Registration

In order to facilitate planning for this meeting, persons wishing to attend are asked to register by April 14, 2009, via the NICEATM–ICCVAM Web site (*http://iccvam.niehs.nih.gov/contact/reg_LLNAPanel.htm*). Visitor parking is located in the multi-level parking garage accessible via NIH Gateway Drive. All visitors should proceed to the Gateway Center to receive a visitor badge. Note: parking is limited and a government-issued ID is required for access (an area map, driving directions, and NIH contact information are available at *http://www.nih.gov/about/visitor/index.htm*).

Availability of the Revised Documents

The revised draft BRDs and revised draft ICCVAM test method recommendations will be available from the NICEATM–ICCVAM Web site (*http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm*) by March 3, 2009, or by contacting NICEATM (see **FOR FURTHER INFORMATION CONTACT** above).

Request for Public Comments

NICEATM invites the submission of written comments on the revised draft BRDs and revised draft ICCVAM test method recommendations and prefers that comments be submitted by April 14, 2009, electronically via the NICEATM–ICCVAM Web site *http://iccvam.niehs.nih.gov/contact/FR_pubcomment.htm* or via e-mail at *niceatm@niehs.nih.gov*. Written comments may also be sent by mail, fax, or e-mail to Dr. William Stokes, Director of NICEATM, at the address listed above (see **FOR FURTHER INFORMATION CONTACT**). When submitting written comments, please refer to this **Federal Register** notice and include appropriate contact information (name, affiliation, mailing address, phone, fax, e-mail, and sponsoring organization, if applicable). All comments received will be placed on the NICEATM–ICCVAM Web site (*http://iccvam.niehs.nih.gov*), and identified by the individual's name and affiliation or sponsoring organization (if applicable). Comments will also be provided to the Panel and ICCVAM agency representatives, and made available at the meeting.

Time will be provided for the presentation of oral comments by the public at designated times during the peer review. Members of the public who wish to present oral statements at the meeting (one speaker per organization) should contact NICEATM (see **FOR FURTHER INFORMATION CONTACT** above) by April 14, 2009 and provide a written copy of their comments. Each speaker is asked to provide contact information (name, affiliation, mailing address, phone, fax, e-mail, and sponsoring organization, if applicable) when registering to make oral comments. Up to seven minutes will be allotted per speaker. If this is not possible, please bring 40 copies of your comments to the meeting for distribution and to supplement the record. Written statements can supplement and expand the oral presentation. Please provide NICEATM with copies of any supplementary written statement using the guidelines outlined above.

Summary minutes and the Panel's final report will be available following the meeting on the NICEATM–ICCVAM Web site (*http://iccvam.niehs.nih.gov*). ICCVAM will consider the Panel's conclusions and recommendations and any public comments received in finalizing their test method recommendations and performance standards for these methods.

Background Information on ICCVAM, NICEATM, and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

ICCVAM is an interagency committee composed of representatives from 15 Federal regulatory and research agencies that use, generate, or disseminate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability and promotes the scientific validation and regulatory acceptance of toxicological test methods that more accurately assess the safety and hazards of chemicals and products and that refine, reduce, and replace animal use. The ICCVAM Authorization Act of 2000 (42 U.S.C. 285*l*–3) established ICCVAM as a permanent interagency committee of the NIEHS under NICEATM. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of U.S. Federal agencies. Additional information about ICCVAM and NICEATM can be found on their Web site (*http://iccvam.niehs.nih.gov*).

SACATM was established January 9, 2002, and is composed of scientists from the public and private sectors (67 FR 11358). SACATM provides advice to the Director of the NIEHS, to ICCVAM, and to NICEATM regarding the statutorily-mandated duties of ICCVAM and activities of NICEATM. Additional information about SACATM, including the charter, roster, and records of past meetings, can be found at *http://ntp.niehs.nih.gov/*; see ''Advisory Board & Committees'' (or directly at *http://ntp.niehs.nih.gov/go/167*).

Dated: February 19, 2009.

John R. Bucher,

Associate Director, NTP.

[FR Doc. E9–4280 Filed 2–26–09; 8:45 am]

BILLING CODE 4140–01–P

DEPARTMENT OF HEALTH AND HUMAN SERVICES

National Toxicology Program (NTP); Office of Liaison, Policy and Review; Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

AGENCY: National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH).

ACTION: Meeting announcement and request for comments.

SUMMARY: Pursuant to section 10(a) of the Federal Advisory Committee Act, as amended (5 U.S.C. Appendix 2), notice is hereby given of a meeting of SACATM on June 25–26, 2009, at the Hilton Arlington Hotel, 950 North Stafford Street, Arlington, VA 22203. The meeting is open to the public with attendance limited only by the space available. SACATM advises the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and the Director of the NIEHS and NTP regarding statutorily mandated duties of ICCVAM and activities of NICEATM.

DATES: The SACATM meeting will be held on June 25 and 26, 2009. The meeting is scheduled from 8:30 a.m. to 5 p.m. on June 25 and 8:30 a.m. until adjournment on June 26, 2009. All individuals who plan to attend are encouraged to register online at the NTP Web site (*http://ntp.niehs.nih.gov/go/7441*) by June 17, 2009. In order to facilitate planning, persons wishing to make an oral presentation are asked to notify Dr. Lori White, NTP Executive Secretary, via online registration, phone, or e-mail by June 17, 2009 (see **ADDRESSES** below). Written comments should also be received by June 17, 2009, to enable review by SACATM and NIEHS/NTP staff before the meeting.

ADDRESSES: The SACATM meeting will be held at the Hilton Arlington Hotel, 950 North Stafford Street, Arlington, VA 22203 [hotel: (703) 528–6000)]. Public comments and other correspondence should be directed to Dr. Lori White (NTP Office of Liaison, Policy and Review, NIEHS, P.O. Box 12233, MD K2–03, Research Triangle Park, NC 27709; telephone: 919–541–9834 or e-mail: *whiteld@niehs.nih.gov*). Courier address: NIEHS, 530 Davis Drive, Room 2136, Durham, NC 27713. Persons needing interpreting services in order to attend should contact 301–402–8180 (voice) or 301–435–1908 (TTY).

Requests should be made at least 7 days in advance of the meeting.

SUPPLEMENTARY INFORMATION:

Preliminary Agenda Topics and Availability of Meeting Materials

Preliminary agenda topics include:

- NICEATM–ICCVAM Update.
- Regulatory Acceptance of ICCVAM–Recommended Alternative Test Methods.
- NRC Report *Recognition and Alleviation of Pain in Laboratory Animals*.
- Implementation of NICEATM–ICCVAM Five-Year Plan.
- Federal Agency Research, Development, Translation, and Validation Activities Relevant to the NICEATM–ICCVAM Five-Year Plan (EPA and USDA).
- Report on second meeting of Independent Peer Review Panel: Evaluation of the Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products.
- Report on the Independent Scientific Peer Review Panel on Alternative Ocular Safety Testing Methods.
- Update from the Japanese Center for the Validation of Alternative Methods.
- Update from the European Centre for the Evaluation of Alternative Methods.
- Update from Health Canada.

A copy of the preliminary agenda, committee roster, and additional information, when available, will be posted on the NTP Web site (*http://ntp.niehs.nih.gov/go/7441*) or available upon request (see **ADDRESSES** above). Following the SACATM meeting, summary minutes will be prepared and available on the NTP Web site or upon request.

Request for Comments

Both written and oral public input on the agenda topics is invited. Written comments received in response to this notice will be posted on the NTP Web site. Persons submitting written comments should include their name, affiliation (if applicable), and sponsoring organization (if any) with the document. Time is allotted during the meeting for presentation of oral comments and each organization is allowed one time slot per public comment period. At least 7 minutes will be allotted for each speaker, and if time permits, may be extended up to 10 minutes at the discretion of the chair. Registration for oral comments will also be available on-site, although time allowed for presentation by on-site registrants may be less than for pre-registered speakers and will be determined by the number of persons who register at the meeting.

Persons registering to make oral comments are asked to do so through the online registration form (*http://ntp.niehs.nih.gov/go/7441*) and to send a copy of their statement to Dr. White (see **ADDRESSES** above) by June 17, 2009, to enable review by SACATM, NICEATM–ICCVAM, and NIEHS/NTP staff prior to the meeting. Written statements can supplement and may expand the oral presentation. If registering on-site and reading from written text, please bring 40 copies of the statement for distribution and to supplement the record.

Background Information on ICCVAM, NICEATM, and SACATM

ICCVAM is an interagency committee composed of representatives from 15 Federal regulatory and research agencies that use, generate, or disseminate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability and promotes the development, scientific validation, regulatory acceptance, implementation, and national and international harmonization of new, revised, and alternative toxicological test methods that more accurately assess the safety and hazards of chemicals and products and that refine, reduce, and replace animal use. The ICCVAM Authorization Act of 2000 [42 U.S.C. 285*l*–3] established ICCVAM as a permanent interagency committee of the NIEHS under NICEATM. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of U.S. Federal agencies. Additional information about ICCVAM and NICEATM can be found on their Web site (*http://iccvam.niehs.nih.gov*).

SACATM was established in response to the ICCVAM Authorization Act [Section 285*l*–3(d)] and is composed of scientists from the public and private sectors. SACATM advises ICCVAM, NICEATM, and the Director of the NIEHS and NTP regarding statutorily mandated duties of ICCVAM and activities of NICEATM. SACATM provides advice on priorities and activities related to the development, validation, scientific review, regulatory acceptance, implementation, and national and international harmonization of new, revised, and alternative toxicological test methods. Additional information about SACATM, including the charter, roster, and records of past meetings, can be found at *http://ntp.niehs.nih.gov/go/167*.

Dated: April 22, 2009.

John R. Bucher,

Associate Director, National Toxicology Program.

[FR Doc. E9–9845 Filed 4–28–09; 8:45 am]

BILLING CODE 4140–01–P

DEPARTMENT OF HEALTH AND HUMAN SERVICES

National Toxicology Program (NTP); NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

AGENCY: National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH).

ACTION: Request for comments.

SUMMARY: NICEATM, in collaboration with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), convened an independent, international, scientific peer review panel (hereafter, Panel) on April 28–29, 2009, to evaluate three non-radioactive modified versions and new applications for the Murine Local Lymph Node Assay (LLNA). The LLNA is an alternative test method that can be used to determine the allergic contact dermatitis potential of chemicals and products. The Panel report from this meeting is now available. The report contains (1) the Panel's evaluation of the updated validation status of the methods and (2) the Panel's comments on the updated draft ICCVAM test method recommendations. NICEATM invites public comment on the Panel's report. The report is available on the NICEATM–ICCVAM Web site at *http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2009.pdf* or by contacting NICEATM at the address given below.

DATES: Written comments on the Panel report should be received by July 15, 2009.

ADDRESSES: Comments should be submitted preferably electronically via the NICEATM–ICCVAM Web site at *http://iccvam.niehs.nih.gov/contact/FR_pubcomment.htm*. Comments can also be submitted by e-mail to *niceatm@niehs.nih.gov*. Written comments can be sent by mail or fax to Dr. William S. Stokes, Director, NICEATM, NIEHS, P.O. Box 12233, *Mail Stop:* K2–16, Research Triangle Park, NC 27709; (fax) 919–541–0947. *Courier address:* NIEHS, NICEATM, 530 Davis Drive, Room 2035, Durham, NC 27713.

FOR FURTHER INFORMATION CONTACT: Dr. William S. Stokes (telephone) 919–541–2384, (fax) 919–541–0947 and (e-mail) *niceatm@niehs.nih.gov*.

SUPPLEMENTARY INFORMATION:

Background

In January 2007, the Consumer Product Safety Commission submitted a nomination to NICEATM and ICCVAM to assess the validation status of (1) the use of the LLNA to determine potency for hazard classification purposes, (2) LLNA protocols using non-radioactive procedures, (3) the LLNA limit dose procedure, and (4) the use of the LLNA to test mixtures, aqueous solutions, and metals (*i.e.*, an updated assessment of the applicability domain of the LLNA). In June 2007, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) endorsed these activities as high priorities for ICCVAM. NICEATM, on behalf of ICCVAM, also sought input from the public on these activities and requested data from studies using the LLNA or modified versions of the LLNA (72 FR 27815). After considering all comments received, ICCVAM endorsed carrying out these activities as high priorities. ICCVAM also developed draft LLNA performance standards to facilitate evaluation of modified LLNA protocols that are functionally and mechanistically similar to the traditional LLNA. These draft LLNA performance standards were made public and comments were requested in September 2007 (72 FR 52130).

ICCVAM and NICEATM prepared draft background review documents (BRDs) that provided comprehensive reviews of available data and relevant information for each of the modifications and new applications of the LLNA. ICCVAM also developed draft test method recommendations regarding the proposed usefulness and limitations, standardized protocols, and future studies. NICEATM announced availability of the draft BRDs and draft recommendations for public comment and the public peer review meeting in January 2008 (73 FR 1360).

The Panel met in public session on March 4–6, 2008, to review these topics, and their report was made available in May 2008 (73 FR 29136). The draft BRDs and draft test method recommendations, the draft ICCVAM LLNA test method performance standards, the Panel's report, and all public comments were made available to SACATM for comment at their meeting on June 18–19, 2008 (73 FR 25754).

As a result of additional data received by ICCVAM subsequent to the March 2008 Panel meeting, the draft BRDs for the following were updated:

- The validation status of three modified LLNA test method protocols that do not require the use of radioactive substances.
- The use of the LLNA for testing pesticide formulations, other products, and aqueous solutions.

Second Meeting of the Peer Review Panel

The Panel met again in public session on April 28–29, 2009 (74 FR 8974). The Panel reviewed the revised draft ICCVAM documents for completeness, errors, and omissions of any existing relevant data or information. The Panel evaluated the information in the revised draft documents to determine the extent to which each of the applicable criteria for validation and acceptance of toxicological test methods (ICCVAM, 2003) had been appropriately addressed. The Panel then considered the ICCVAM draft recommendations for test method uses and limitations, proposed standardized protocol, proposed plans for development of test method performance standards, and proposed additional studies, and commented on the extent that the recommendations were supported by the information provided in the draft BRDs.

Availability of the Peer Panel Report

The Panel's conclusions and recommendations are detailed in the

Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products (available at *http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2009.pdf*). The revised draft documents reviewed by the Panel and the draft ICCVAM test method recommendations are available at *http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm*.

Request for Public Comments

NICEATM invites the submission of written comments on the Panel's report. When submitting written comments, please refer to this **Federal Register** notice and include appropriate contact information (name, affiliation, mailing address, phone, fax, e-mail, and sponsoring organization, if applicable). All comments received will be made publicly available via the NICEATM–ICCVAM Web site at *http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm*. In addition, there will be an opportunity for oral public comments on the Panel's report during an upcoming meeting of SACATM scheduled for June 25–26, 2009 (74 FR 19562). Information concerning the SACATM meeting is available at *http://ntp.niehs.nih.gov/go/7441*. ICCVAM will consider the Panel report along with SACATM and public comments when finalizing test method recommendations. An ICCVAM test method evaluation report, which will include the final ICCVAM recommendations, will be forwarded to relevant Federal agencies for their consideration. The evaluation report will also be available to the public on the NICEATM–ICCVAM Web site at *http://iccvam.niehs.nih.gov/methods/immunotox/llna.htm* and by request from NICEATM (see **ADDRESSES** above).

Background Information on ICCVAM, NICEATM, and SACATM

ICCVAM is an interagency committee composed of representatives from 15 Federal regulatory and research agencies that use, generate, or disseminate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability and promotes the scientific validation and regulatory acceptance of toxicological test methods that more accurately assess the safety and hazards of chemicals and products and that refine, reduce, and replace animal use. The ICCVAM Authorization Act of 2000 (42 U.S.C. 285*l*–3) established ICCVAM as a permanent interagency committee of the NIEHS under NICEATM. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of U.S. Federal agencies. Additional information about ICCVAM and NICEATM can be found on their Web site (*http://iccvam.niehs.nih.gov*).

SACATM was established January 9, 2002, and is composed of scientists from the public and private sectors (67 FR 11358). SACATM provides advice to the Director of the NIEHS, ICCVAM, and NICEATM regarding the statutorily mandated duties of ICCVAM and activities of NICEATM. Additional information about SACATM, including the charter, roster, and records of past meetings, can be found at *http://ntp.niehs.nih.gov/* see ''Advisory Board & Committees'' (or directly at *http://ntp.niehs.nih.gov/go/167*).

Reference

ICCVAM. 2003. ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods. NIH Publication No. 03–4508. Research Triangle Park, NC: NIEHS. Available at: *http://iccvam.niehs.nih.gov.*

Dated: May 19, 2009.

John R. Bucher,

Associate Director, NTP.

[FR Doc. E9–12360 Filed 5–29–09; 8:45 am]

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Appendix F2

Public Comments Received in Response to *Federal Register* Notices

72 FR 27815 (May 17, 2007)
The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data

- Dr. Eric Debruyne (BAYER CropScience) F-27
- Dr. H.-W. Vohr (Bayer HealthCare AG) F-29
- Dr. H.-W. Vohr (Bayer HealthCare AG) F-34
- Dr. H.-W. Vohr (Bayer HealthCare AG) F-39
- Dr. Kirill Skirda (CESIO) F-44
- Mark S. Maier, Ph.D., DABT (CropLife America) F-45
- Dr. Phil Botham (European Crop Protection Association) F-46
- Peter Ungeheuer (European Federation for Cosmetic Ingredients) F-49
- Dori Germolec (NIEHS) F-51
- Dori Germolec (NIEHS) F-52
- Robert L. Guest (Safepharm Laboratories Ltd) F-53
- Daniel R. Cerven, M.S. and Melissa K. Kirk, Ph.D. (MB Research Laboratories) F-55
- Daniel Marsman, D.V.M., Ph.D. (Procter & Ganble) F-59
- Michael J. Olson, Ph.D. (GlaxoSmithKline) F-60
- Anne Marie Api, Ph.D. (Research Institute for Fragrance Manufacturers) F-62
- Peter S. Thorne, Ph.D. (The University of Iowa) F-64
- Catherine Willett, Ph.D. (People for the Ethical Treatment of Animals), Sara Amundson (Humane Society Legislative Fund), Dr. Martin Stephens (Humane Society of the United States), Kristie Stoick, M.P.H. (Physicians Committtee for

Responsible Medicine), Sue A. Leary (Alternatives Research & Development Foundation), and Tracie Letterman, Esq. (American Anti-Vivisection Society) F-65

72 FR 52130 (September 12, 2007)
Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments

- Ann-Therese Karlberg (Goteborg University) F-70
- Dr. Jon Richmond F-71
- Prof. dr. Henk Van Loveren (National Institute of Public Health and the Environment, the Netherlands) F-73
- Catherine Willett, Ph.D. (People for the Ethical Treatment of Animals), Sara Amundson (Humane Society Legislative Fund), Dr. Martin Stephens (Humane Society of the United States), Kristie Stoick, M.P.H. (Physicians Committee for Responsible Medicine), Sue A. Leary (Alternatives Research & Development Foundation), and Tracie Letterman, Esq. (American Anti-Vivisection Society) F-75

73 FR 1360 (January 8, 2008)
Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments

- Dr. David Basketter F-78
- Dr. David Basketter F-79
- Kenneth T. Bogen, Dr.P.H., DABT (Exponent) F-80
- G. Frank Gerberick, Ph.D. (The Procter & Gamble Company) F-81
- Laurence Musset (OECD) F-90
- B. Schau F-93
- Catherine Willett, Ph.D. (People for the Ethical Treatment of Animals) and Kristie Stoick, M.P.H. (Physicians Committee for Responsible Medicine) F-94

73 FR 25754 (May 7, 2008)
Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

- B. Sachau F-99

73 FR 29136 (May 20, 2008)
Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

- No responses received

74 FR 8974 (February 27, 2009)
Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments

- Nancy Douglas, Ph.D. and Catherine Willett, Ph.D. (People for the Ethical Treatment of Animals), Kristie Stoick, M.P.H. (Physicians Committee for Responsible

Medicine), Martin Stephens, Ph.D. (The Humane Society of the United States), Sara Amundson (Humane Society Legal Fund, Doris Day Animal League), Sue Leary (Alternatives Research & Development Foundation), and Tracie Letterman, Esq. (American Anti-Vivisection Society) F-100

74 FR 19562 (April 29, 2009)
Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

- No responses received

74 FR 26242 (June 1, 2009)
Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

- Brian E. Harvey, M.D., Ph.D. (Sanofi Aventis) F-105

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Date: Fri, 29 Jun 2007 09:56:56 +0200
To: Neepa Choksi
Cc: David Allen, Doug Winters
Subject: Re: RE ICCVAM/NICEATM FR Notice: LLNA Nomination and Request for Data

Dear Neepa,

In response to the NICEATM request published in the Federal Register notice, vol. 72, N° 95, on May 17th, 2007 and further to the e-mails we have exchanged on this subject, Bayer CropScience is submitting data from a number of studies conducted using the LLNA assay with different types or pesticide formulations. This data is submitted specifically to address the following questions: (1) the evaluation of the LLNA as a stand-alone assay for determining potency for the purpose of hazard classification, and (2) the ability of the LLNA for testing mixtures and aqueous solutions".

In our studies, the LLNA study protocol includes the addition of a positive control spiked into the tested formulation in order to demonstrate the ability of the assay to detect sensitizer in such formulations and thus the validity of the results.

The data is submitted in two forms in the attached zipped file:
1. detailed summaries of the data obtained with several formulations using both the LLNA and another validated method for evaluation of the sensitization potential of the pesticide formulation (Buehler tests with 3 or 9 inductions, Maximized M&K test) are provided for 11 different pesticide formulations (EC, SL, EW, OF, WG, SC).
2. full reports of most of the studies from the above list where the LLNA assay showed a positive response while the classical methods were negative.

Please note that, for confidentiality reasons, the names of the active ingredients contained in the different formulations have been blinded.

We hope that this data will be useful to the evaluation conducted by the NICEATM. Please do not hesitate to use me as your contact for any queries or questions on our data and studies.

Cordialement / Best regards / Mit freundlichen Grüßen

Eric

_____

Dr Eric DEBRUYNE
BAYER CropScience
Head of Experimental Toxicology
Sophia Antipolis Research Centre

355, rue Dostoievski - BP153
F-06903 Sophia Antipolis Cedex
FRANCE
Phone: +33 (0)4 92 94 34 37 (direct line)
Assistant: +33 (0)4 92 94 34 80 (Marie-France)
FAX: +33 (0)4 93 95 84 54
Cell phone: +33 (0)6 72 75 92 67
E-Mail: eric.debruyne@bayercropscience.com

Bayer HealthCare

Neepa Y. Choksi, Ph.D.

Senior Staff Toxicologist

Integrated Laboratory Systems, Inc./Contractor
supporting the NTP Interagency Center for the
Evaluation of Alternative Toxicological Methods
National Institute of Environmental Health Sciences

MD EC-17, P.O. BOX 12233

Research Triangle Park, NC 27709

Bayer HealthCare AG

PH-PD Toxikologie
Prof. Dr. H.-W. Vohr
D - 42096 Wuppertal
Telefon: 0202 - 36 8811
Telefax: 0202 - 36 4137
E-Mail: hans-werner.vohr@bayerhealthcare.com

6/20/07

Concerns:
Data package 1, submitted to NICEATM and ICCVAM for further evaluation of the LLNA and modifications of it

In 2001 experts of several institutes (authority, academia, industry) in Europe decided to initiate a catch-up validation of a modification of the standard - radioactive - LLNA as described before by Homey et al. and Vohr et al. [Ref. 1.1 and 1.2.]. From the very beginning the studies were supported by the VCI (Verband der Chemischen Industrie e.V. (German chemical industry federation)).

It was decided to test 3 (first round) and 9 (second round) international standards out of a list of 26 standards under full GLP compliance. The substances should be submitted blinded by an independent coordinator to the participating labs. A well-known expert from the Swiss authority Swissmedic, T. Maurer, accepted to supervise the study, to select the test substances including submission of the test items as well as to organize the data submission to an independent statistician (J. Hüsler, University of Bern, Switzerland).

It was decided to start with a pilot study using HCA as test substance to finally harmonize the protocol used by the participating labs. In addition, a new evaluation scheme was agreed on which takes the assessment of skin reaction due to irritation into account [Ref. 1.3.].

Afterwards a first round with 3 test substances and two strains of mice (BALB/c and NMRI outbred) had been carried out. The test items were not only blinded but also labeled differently for each participating lab for this first part of the study by the coordinator. An intermediate assessment of the still blinded test substances served as a milestone to continue or not, and to

select one of the mouse strains for the second round of the study. Because of extremely good correlation of the data between labs it was decided to continue with another 9 standards in a second round with BALB/c.

All 9 participating labs measured weights and cell counts of the draining lymph nodes, and for acute skin reaction ear weights (8mm punch). Ear thickness was measured in some labs in addition. One lab used radioactive labeling as well, and one lab used NMRI also with all standards.

All raw data were sent to T. Maurer who forwarded these to J. Hüsler for statistical evaluation [Ref.1.4.]. Only after the overall evaluation the codes were de-blinded by T. Maurer.

Evaluation based on cell count indices turned out to be as sensitive as the radioactive method. The cut-off concentrations (EC values) were very similar for both methods (cf. also publications of the catch-up validation).

The additional determination of acute ear (skin) reaction by ear weight/ear thickness turned out to be very useful for further assessment of the lymph node reaction, i.e. to exclude false positive results. Results of this catch-up validation have been published in peer reviewed papers [Ref 1.5. and 1.6.] and at different meetings in poster sessions.

With respect to the cut-off values (EC (Effective Concentration) values) it is obvious that each parameter (end point) requires its own specific cut-off value. This is accepted since decades for example in guinea pig assays: >= 30% positive reactions in M&K tests or >= 15% positive reactions in Bühler tests.

For the radioactive labeling the cut-off value has been fixed to that concentration of test substance that induces a 3 times increase in stimulation index, i.e. the so-called EC3 value. For cell count indices such cut-off values are much lower, for example 1.5 times increase of stimulation index. This is understandable by the facts that cell count indices have i) lower individual variances compared to 3H-Thymidine incorporation, and ii) lower maximum stimulation indices compared to radioactive labeling. For example, a strong sensitizing substance may easily induce indices about 30-50 by 3H-Thymidine incorporation but only indices about 4-5 by cell counting. However, crucial for the assessment are not impressive high stimulation indices, but reliable determination of a safe and accurate cut-off value, so the reasonable and reliable determination of the concentration of a test substance exceeding it. These concentrations exceeding the thresholds can then be compared between methods and modifications, and are indeed comparable as it has been shown by our catch-up validation! In [Ref. 1.7.] the results of EC1.5 values of all participating labs are averaged and the classification range of potency given as calculated in the different labs. Statistically significant increases were taken into account just as all stimulation indices exceeding the cut-off value. i.e. EC1.5, without being of statistical significance.

Interestingly, there was an extremely good correlation between statistically significant increases in stimulation indices and the exceeding of thresholds or cut-off values. Similar finding have already been published by Gerberick et al. in 1992 [Ref. 1.8.] as can be taken from the attached table (statistically significant indices in red):

Table 1 (modified after Gerberick et al., 1992) showing significant stimulation indices of two different endpoints, i.e. cell counting or radioactive labeling obtained with international standards.

Compound	Cell counts	3H.Thymidine
Benzalkonium chloride 0,5%	2,70	9,00
1%	4,08	11,10
2%	2,93	7,60
Benzocaine 5%	1,39	1,30
10%	0,99	1,00
20%	1,12	1,30
DCNB 0,001%	0,94	0,80
0,05%	2,06	10,70
0,10%	2,83	21,10
Ethylendiamine 1%	1,06	1,10
5%	1,07	1,10
10%	1,77	2,20
Eugenol 25%	2,72	5,40
50%	2,70	10,60
75%	2,72	10,50
Glutaraldehyde 3,1%	2,54	9,80
6,20%	4,52	21,40
12,50%	5,35	22,90
MCI/MI 50ppm	3,04	8,10
500ppm	5,68	27,80
1000ppm	4,59	48,20
Nickel cloride 2,5%	0,98	1,30
5%	1,50	2,60
10%	1,96	6,60
Oxazolone 0,0001%	0,94	1,60
0,005%	1,62	8,70
0,05%	4,52	55,20
TNCB 0,01%	3,02	18,00
0,05%	6,62	80,30
0,10%	7,23	103,30

Beside all references mentioned here in the text two reports with all standards tested in one lab with BALB/c or NMRI (outbred) mice are also included in this package 1. Of course, the test substances are called in both reports A to L, but A to C were differently named in each participating lab.

The actual identity of these standards can be taken from the following Table 2:

Round I

Code	Compound	Proposed classification	Reference
HCA	Hexylcinnamaldehyde	Sensitiser	Dearman 2001
A	p-hydroquinone	Sensitiser	Kimber 1998
B	SDS	Irritant	Basketter 1992
C	4-aminobenzoic acid	Negative	Basketter 1992

Round II

Code	Compound	Proposed classification	Test concentrations	Reference
D	Xylene	Irritant	10, 30, 100%	Kligman 1966
E	Octanoic acid	Weak Irritant	1, 3, 10%	ECETOC 1995
F	MCI	Sensitiser	0.03, 0.1, 0.3%	Botham 1991
G	Mercaptobenzothiazole*	Sensitiser	3, 10, 30%	Scholes 1992
H	Isoeugenol	Sensitiser	3, 10, 30%	Basketter 1992
I	Potassium dichromate	Sensitiser	0.3, 1, 3%	Basketter 1992
K	Hydroxycitronellal	Sensitiser	6, 20, 60%	Basketter 1992 Montelius 1994
L	Tween 80	Irritant	10, 30, 100%	Magnusson 1969

Kind regards,

H.-W. Vohr

References:

1.1. Homey, B, von Schilling, C., Blümel, J., Schuppe, H.-C., Ruzicka, T., Ahr, H.-J., Lehmann, P. and Vohr, H.-W.
An Integrated Model for the Differentiation of Chemical-induced Allergic and Irritant Skin Reactions
Toxicol. Appl. Pharmacol. **153**, 83-94, 1998

1.2. Vohr, H.-W., Blümel, J., Blotz, A., Homey, B. and Ahr, H.J.
An intra-laboratory validation of IMDS: Discrimination Between (Photo)Allergic and (Photo)Irritant Skin Reactions in Mice.
Arch. Toxicol., 73, 501-509, 2000

1.3. Protocol of the kick-off meeting of the European catch-up validation study of the modified LLNA

1.4. First statistical evaluation of the second round (test substances D-L) of the catch-up validation by J. Hüsler, Bern, Switzerland

1.5. Ehling G, Hecht M, Heusener A, Huesler J, Gamer AO, v. Loveren, H., Maurer Th, Riecke K, Ullmann L, Ulrich P, Vandebriel R, Vohr H-W
An European Inter-Laboratory Validation of Alternative Endpoints of the Murine Loacl Lymph Node Assay. First ROUND.
Toxicology, 212, 60-68, 2005

1.6. Ehling G, Hecht M, Heusener A, Huesler J, Gamer AO, v. Loveren, H., Maurer Th, Riecke K, Ullmann L, Ulrich P, Vandebriel R, Vohr H-W
An European Inter-Laboratory Validation of Alternative Endpoints of the Murine Loacl Lymph Node Assay. 2nd ROUND.
Toxicology, 212, 69-79, 2005

1.7. Table (PP) showing the classification of the standards tested in the catch-up validation based on different end points and methods according the ECETOC Technical report No. 87, 2003.

1.8. Gerberick GF, House RV, Fletcher ER, Ryan CA
Examination of the Local Lymph Node Assay for Use in Contact Sensitization Risk Assessment
Fundamental and Applied Toxicology, 19,438-445, 1992

Bayer HealthCare

Neepa Y. Choksi, Ph.D.

Senior Staff Toxicologist

Integrated Laboratory Systems, Inc./Contractor
supporting the NTP Interagency Center for the
Evaluation of Alternative Toxicological Methods
National Institute of Environmental Health Sciences

MD EC-17, P.O. BOX 12233

Research Triangle Park, NC 27709

Bayer HealthCare AG

PH-PD Toxikologie
Prof. Dr. H.-W. Vohr
D - 42096 Wuppertal
Telefon: 0202 - 36 8811
Telefax: 0202 - 36 4137
E-Mail: hans-werner.vohr@bayerhealthcare.com

6/20/07

Concerns:
Data package 2, submitted to NICEATM and ICCVAM for further evaluation of the LLNA and modifications of it

In 2005 the BG Institute for Occupational Safety and Health of the German Social Accident Insurance - BGIA (Berufsgenossenschaftliches Institut für Arbeitsschutz) initiated a meeting about skin sensitization, and the experiences so far with the Local Lymph Node Assay. Experts from different institutes (authority, academia, industry) in Germany discussed the data. There was a concern about the increase in positive results with LLNA compared to the years of experiences with guinea pig assays. This is also illustrated by the peer reviewed paper of Vohr and Ahr, 2005 [Ref. 2.1.]. During this meeting it was decided to compare the "standard" - radioactive - LLNA with a non-radioactive modification, i.e. cell counting, with 13 related compounds (epoxy resin components); most of which are classified as skin sensitizers based on guinea pig data. HCA was chosen as positive control. In accordance with the exemplary described method in OECD 429 mouse strain CBA was used for this study. For further information about the compounds and protocol see also Ref 2.2. and 2.3., and Table 1 below. Although both PP presentations are in German the main messages are clear and self-explanatory.

One of the goals was to correlate stimulation indices of both methods as well as cut-off concentrations evaluated by them, i.e. the effective or estimated concentrations of test items exceeding the cut-off lines defined for both methods. These EC values correspond to EC3 for the radioactive labeling or EC1.5 for the cell counting as also described previously [Ref. 2.4.].

Another aim of this study was to classify the test substances according to their potency to induce cell proliferation in the draining lymph nodes. This classification was based on the ECETOC

criteria described before [Ref. 2.5.]. Due to the fact that applications of moderate to strong irritants could result in false positive reactions ear weight was measured in addition to balance the influence of such non-specific cell activation. It has to be mentioned, however, that here skin reactions were measured three days after the last application (on day 6) while the "acute" skin reaction has reasonably to be measured one day after the last application on day 4. In case of 6 days protocols this parameter could be determined by measuring ears swelling at day 4 which was unfortunately not possible during this study. However, this has no influence on the overall assessment of the results, esp. on the comparison of estimated concentrations and stimulation indices.

Following 13 related test substances have been chosen for the comparison (Table 1):
Acetone was used as vehicle to reach acceptable solubility for all test items. Therefore, the positive control HCA was also tested in acetone.

Bisphenol A, resin, Bakelite EPR 164 (CAS-Nr. 25068-38-6)
Bisphenol A, resin, distilled, Bakelite EPR 162 (CAS-Nr.1675-54-3)
Bisphenol F, resin, Bakelite EPR 161 (CAS-Nr. 9003-36-5)

1,6-Hexanediol Diglycidyl Ether (CAS-Nr. 16096-31-4)
P-Tertbutylphenyl Glycidyl Ether (CAS-Nr. 3101-60-8)
Trimethylolpropane triglycidyl ether (CAS-Nr. 3454-29-3)
Dodecyl/tetradecyl glycidyl ether (CAS-Nr. 68609-97-2)

m-Xylylenediamine (CAS-Nr. 1477-55-0)
3-Aminomethyl-3,5,5-trimethylcyclohexylamine (CAS-Nr. 2855-13-2)
Bis(3-aminopropyl)amine (CAS-Nr. 56-18-8)
2,2,4(2,4,4)-Trimethyl-1,6-hexanediamine (CAS-Nr. 25620-58-0)
N-(2-Hydroxyethyl)ethylenediamine (CAS-Nr. 111-41-1)
1,2-Diaminocyclohexane (CAS-Nr. 694-83-7)

All the studies have been conducted at BASF AG, Ludwigshafen, Germany, under full GLP compliance. Data were presented by the study director, Dr. A.O. Gamer, and discussed in a similar panel as before.

Conclusions:

--- There was an extremely good correlation between stimulation indices obtained by radioactive labeling and non-radioactive cell counting [see also Fig. 1 below and Ref. 2.6.].

--- Therefore, the effective concentrations calculated are very similar for both endpoints [see also Table 2 below and Ref. 2.6.].

--- The vehicle (acetone) may have an impact in the relatively low effective doses (i.e. relative high potency) determined for the test substances. This may easily be recognized by the results obtained with HCA diluted in acetone alone or acetone:olive oil (AOO 4:1).

--- Taken the irritant potential also into account will improve the assessment of the overall sensitizing potency. However, optimal time point for the determination of acute skin reaction is one day after last application, i.e. day 4 in standard protocol.

Figure 1: Comparison of lymph node cell count and ^{3}H-thymidine incorporation taken from the Report by AO Gamer and R Landsiedel [Ref 2.6.]

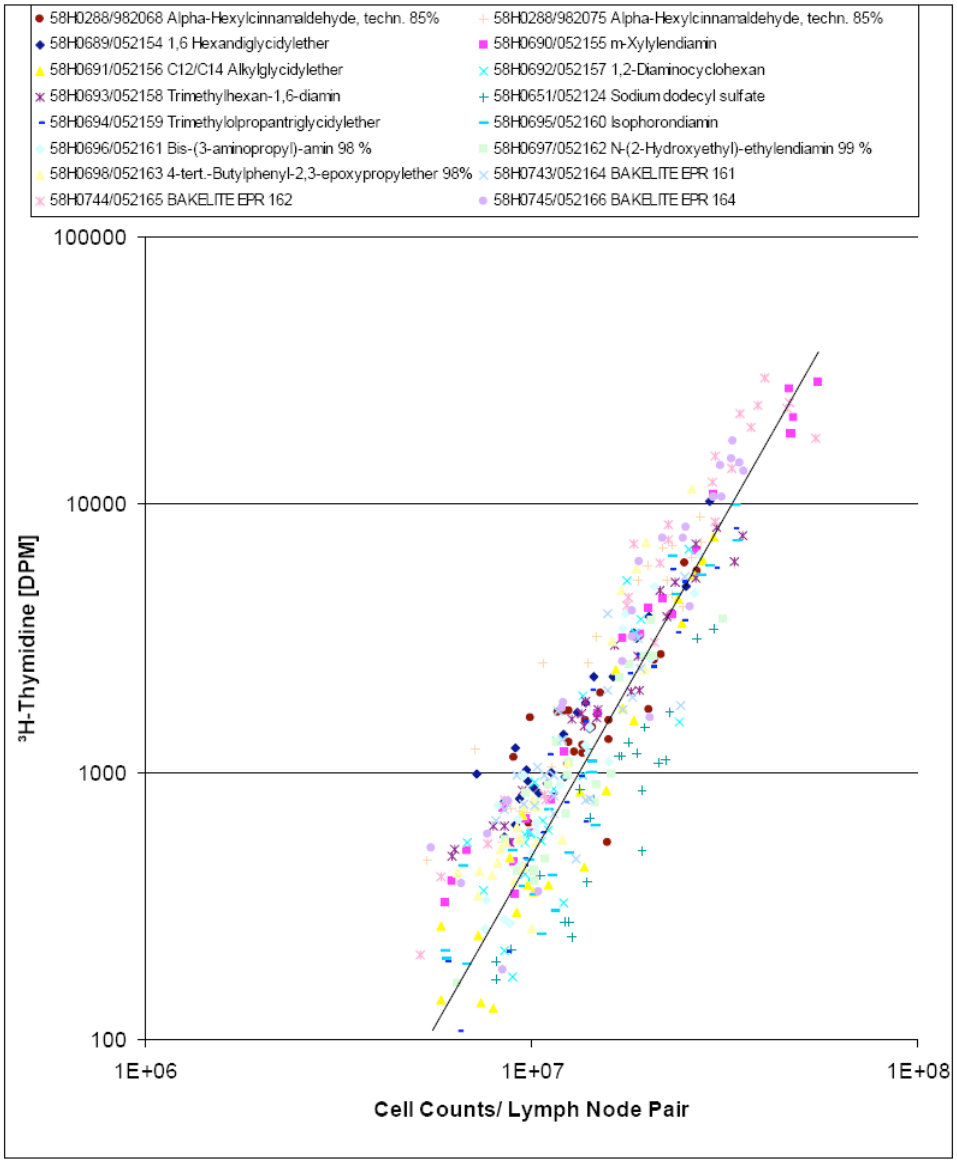


Table 2: Tested concentrations and "Estimated Concentrations[1]" of skin sensitising threshold of epoxy resin components from Ref. 2.6.

Substance name	Concentrations tested	Results			
	% (w/w)	EC3	EC1.5	EC3lg	EC1.5lg
1,6-Bis(2,3-epoxypropoxy)hexane	3, 1, 0.3	1.9	1.7	1.6	1.5
m-Phenylenebis(methylamine)	3, 1, 0.3	0.4	0.4	0.4	0.3
Oxirane, mono((C12-14-alkyloxy)methyl)derivs	3, 1, 0.3	0.6	0.7	0.5	0.6
1,2-Diaminocyclohexane	1, 0.3, 0.1	0.4	0.6	0.4	0.5
Trimethylhexamethylene diamine	10, 3, 1	1.9	0.5	1.7	0.8
1-(2,3-Epoxypropoxy)-2,2-bis[(2,3-epoxypropoxy)methyl]butane	10, 3, 1	1.4	1.7	1.3	1.4
3-Aminomethyl-3,5,5-trimethylcyclohexylamine	3, 1, 0.3	1.0	1.2	1.0	1.1
Dipropylene triamine 98%	3, 1, 0.3	0.9	1.0	0.8	1.0
N-(2-Hydroxyethyl)-ethylendiamine 99%	30, 10, 3	15.2	14.4	13.3	12.7
p-tert-Butylphenyl 1-(2,3-epoxy)propyl ether 98%	1, 0.3, 0.1	0.4	0.5	0.3	0.4
Bakelite EPR 161	1, 0.3, 0.1	0.7	0.6	0.6	0.5
Bakelite EPR 162	3, 1, 0.3	-	-	0.2	0.1
Bakelite EPR 164	3, 1, 0.3	0.1	-	0.2	0.1
α-Hexyl cinnamic aldehyde/AOO	10, 5, 2.5	10.5	6.9	10.7	6.5
α-Hexyl cinnamic aldehyde/Acetone	30, 10, 3	-	0.1	1.2	1.8

- no meaningful calculation possible

[1] Estimated concentration that leads to the respective stimulation index
EC was estimated by linear regression
EClg was estimated by linear regression using a log transformation of the concentration

Kind regards,

H.-W. Vohr

References:

2.1. Vohr HW and Ahr HJ.
Sensitivity of the LLNA
Archive of Toxicol. 79, 721-8, 2005

2.2. PP file about the test compounds (epoxy resin components) presented at the kick-off meeting by K. Kefenbaum from BGIA

2.3. PP file about the pros and cons of the Local Lymph Node Assay (LLNA) and test compounds presented at the kick-off meeting by A.O. Gamer from BASF

2.4. Ehling G, Hecht M, Heusener A, Huesler J, Gamer AO, v. Loveren, H., Maurer Th, Riecke K, Ullmann L, Ulrich P, Vandebriel R, Vohr H-W
An European Inter-Laboratory Validation of Alternative Endpoints of the Murine Loacl Lymph Node Assay. 2nd ROUND.
Toxicology, 212, 69-79, 2005

2.5 Basketter, D.A., Butler, M. Gamer, A., Garruige, J.-L., Gerberick, G.F., Kimber, I., Newsome, C., Steiling, W. and Vohr, H.-W.
Contact Sensitisation: Classification According to Potency.
Technical Report No. 78, ECETOC, Brussels (2003).

2.6. Summary report of the GLP studies with all test compounds of the validation described by AO Gamer and R Landsiedel, BASF AG, Germany

Bayer HealthCare

Neepa Y. Choksi, Ph.D.

Senior Staff Toxicologist

Integrated Laboratory Systems, Inc./Contractor
supporting the NTP Interagency Center for the
Evaluation of Alternative Toxicological Methods
National Institute of Environmental Health Sciences

MD EC-17, P.O. BOX 12233

Research Triangle Park, NC 27709

Bayer HealthCare AG

PH-PD Toxikologie
Prof. Dr. H.-W. Vohr
D - 42096 Wuppertal
Telefon: 0202 - 36 8811
Telefax: 0202 - 36 4137
E-Mail: hans-werner.vohr@bayerhealthcare.com

6/28/07

Concerns:
Data package 3, submitted to NICEATM and ICCVAM for further evaluation of the LLNA and modifications of it

The principle of the method had been published in 1989, and a first collaborative validation study in 1991. In these first trials the stimulation of the lymph nodes, i.e. cell proliferation, was measured by ^{3}H-Thymidin incorporation. In 1999 the principle of the LLNA had been stated as valid alternative to guinea pig assays by the ICCVAM, although the need for further modifications was also noted. Concerns focused on false positive results caused by strong irritants or negative results based on the use of aqueous formulations.

In 2002 the method has been published in guideline OECD 429, and 2003 in EPA guideline OPPTS 870.2600 as a stand-alone test. Corresponding to the concerns mentioned above the use of "wholly aqueous vehicles are to be avoided.". As published by Ryan et al. in 2002 1% Pluronic PE 9200 (L92) may be chosen for using aqueous vehicles in the Local Lymph Node Assay [Ref.3.1.]. As can be taken from the information in this paper it is possible to achieve positive results by the addition of this surfactant to aqueous formulations of test items. However, the cut-off concentrations (EC3 values) increased significantly compared to vehicles recommended in the guidelines. Apart from that the data impressively show the influence of vehicles on the cut-off concentrations determined by the LLNA exemplary illustrated by Table 1 (primordial Table 3 in the paper of Ryan et al.).

Table 1 (taken from publication Ryan et al., 2002)

Effect of vehicle on the relative skin sensitization potency of DNBS, formaldehyde, potassium dichromate and nickel sulfate

Chemical	Vehicle	EC3 Value
DNBS	Water	16%
	1% L92	6.4%
	DMSO	2.0%
	DMF	<1.0%
Formaldehyde	Water	14.5%
	1% L92	4.2%
	DMSO	<1.0%
	DMF	<1.0%
Potassium dichromate	1% L92	0.17%
	DMSO	0.05%
	DMF	0.0327%
Nickel sulfate	1% L92	2.5%
	DMSO	4.8%
	DMF	>5.0%

To examine the use of surfactants on the ability to test aqueous formulations in the Local Lymph Node Assay we started with aqueous formulations of HCA. The test item was formulated immediately before each administration in Pluronic PE 9200 / 0.9% NaCl solution, 1% v/v or Cremophor / 0.9% NaCl solution, 2% v/v [cf. also Ref 3.3.].

In a first trial we compared HCA in different vehicles with 2% Cremophor. Results are shown in the Table below (Table 2).

Table 2: Modified LLNA using NMRI and HCA as positive control. Cut-off cell count index is set to 1.4, i.e. EC1.4 should be used [Ref. 3.2.].

HCA					Statist. Signific.		
	Vehicle	**3%**	**10%**	**30%**		**EC1.4**	**Potency***
	MEK	1,22	1.42	1.99	*	9.3	moderate
	AOO (4:1)	1.15	1.28	1.79	*	14.7	weak
	DMF	0.87	1.13	1.77	*	18.4	weak
	PEG400	0.81	1.04	1.69	*	21.1	weak
	Cremophor	0.71	0.98	1.37		(31.5)	(weak)

* Potency classification according to ECETOC technical Report No. 87, 2003

Although an improvement, addition of Cremophor alone did not reach the EC values between 5% and 20% as normally determined with standard (guideline) vehicles. Therefore, we included an additional infrared irradiation (about 20 min. before treatment) of the animals to enhance the blood flow in the skin and by this enhance penetration. This additional treatment by infrared irradiation caused indeed higher, and statistically significant stimulation indices as can be taken from the Table below.

Vehicle	3%	10%	30%	.	EC1.4	Potency#
Cremophor (2%)	0.71	0.98	1.37		(31.5)	(weak)
Cremo. (2%) + IR	0.82	1.34	1.45	*	20.9	weak

*: Statistically significant

#: Potency classification according to ECETOC technical Report No. 87, 2003

Similar studies were then conducted with L92 and infrared irradiation in combination with aqueous HCA formulations. In each case HCA has been classified by this method as weak sensitizer within a range of EC values comparable to those obtained with other (guideline) vehicles. Such positive control studies with aqueous formulations are done in regular intervals in our lab (Bayer HealthCare AG, Immunotoxicology) since years. Results of these studies are also included in the Excel file attached to this data package [Ref. 3.3.].

It has to be mentioned here that based on all our experiences so far with Cremophor or Pluronic it seems that Pluronic (L92) enhances the intrinsic irritant properties of test compounds while Cremophor does not! This property of L92 may be problematic for correct classification of test items when radioactive labeling without discrimination of irritation and sensitization is used for measuring cell proliferation. One example of such a positive control study report with HCA in 1% Pluronic is attached as Ref. 3.4., which is equal to data of Ref. 3.3., "Tabelle 4, 2005/2".

Because sponsors did not want us to submit data with aqueous formulations all we can provide are data from a pre-validation study with HCA as positive controls and three aqueous formulations (A-C) from which one had been tested positive in GPMT before (A as weak sensitizer; B unknown; C tested negative before). The results are given in Ref. 3.5. including all controls with 2% Cremophor or 1% L92 plus infrared irradiation.

The overall conclusion from these studies is that stimulation index induced by formulation A at the highest concentration (50%) just reached the cut-off level of EC1.4, statistically significant. Hence, formulation A would be classified as a weak sensitizing formulation while the other two formulations turned out to be negative.

Conclusions:

--- There is some differences in stimulation indices obtained with various vehicles. EC value may vary by a factor of +/- 2 of overall mean. A change in classification of potency by this factor is possible [cf. also review article by McGarry, 2007; Ref. 3.6.].

--- Aqueous formulations may be tested by adding 1% L92 or 2% Cremophor to the formulation to increase adherence to the skin. Skin irradiation with infrared will accessorily improve the outcome, i.e. test sensitivity.

--- By this modifications (surfactant + infrared irradiation) it is possible to test aqueous formulations with nearly the same sensitivity as with vehicles recommended in the guidelines.

--- However, there is no profound validation study of the LLNA or a modification of it with aqueous formulations or mixtures down to the present day.

--- It seems as if Pluronic enhances the irritant properties of test compounds applied, and by this increase the non-specific activation of lymph node cells which may be a problem for classification according to potency by radioactive methods.

Kind regards,

H.-W. Vohr

References:

3.1. Ryan, C.A., Cruse, L.W., Skinner, R.A., Dearman, R.J., Kimber, I., Gerberick, G.F.. Examination of a vehicle for use with water soluble materials in the murine local lymph node assay.
Food and Chem. Tox. **40** (2002), 1719-1725

3.2. Ehling G, Hecht M, Heusener A, Huesler J, Gamer AO, v. Loveren, H., Maurer Th, Riecke K, Ullmann L, Ulrich P, Vandebriel R, Vohr H-W
An European Inter-Laboratory Validation of Alternative Endpoints of the Murine Loacl Lymph Node Assay. 2nd ROUND.
Toxicology, 212, 69-79, 2005

3.3. Excel file of LLNA-IMDS data with different vehicles including Cremophor and Pluronic, and reliability over the years, Vohr, H-W, Bayer HealthCare AG

3.4. Vohr, H-W, CONFIRMATION OF THE FUNCTION OF A LOCAL LYMPH NODE ASSAY IN MICE (LLNA/IMDS) WITH ALPHA HEXYL CINNAMIC ALDEHYDE
Report PH-34311, T 8075907, 2006

3.5. Brief report (MS word document) of LLNA-IMDS data with different aqueous formulations with Cremophor or Pluronic, Vohr, H-W, Bayer HealthCare AG

3.6. McGarry, The Murine Local Lymph Node Assay: Regulatory and Potency Considerations under REACH.
Toxicology online, 2007 (doi:10.1016/j.tox.2007.06.002)

European Committee of
Organic Surfactants and
their Intermediates

A Sector Group of **Cefic**

12 June 2007

Dr. William S. Stokes
NICEATM Director
NIEHS
Research Triangle Park. NC 27709
Via E-mail: niceatm@niehs.nih.gov

Re. Federal Register 72 (95), May 17, 2007, pages 27815-27817

Dear Dr. Stokes

I am writing to you on behalf the CESIO Local Lymph Node Assay Task Force (CESIO is the Sector Group of CEFIC dealing with organic surfactants and their intermediates). This Task Force was established in 2006 with the aim to exchange the experiences of the different Industry Sectors using the Local Lymph Node Assay (LLNA) for sensitisation testing.

The Task Force noted that several Industry sectors experienced positive results in the LLNA that were unexpected on the basis of the structure activity relation ships (SAR's) or considered false positive results on the basis of guinea pig tests, human experience or other information.

The experience of the Task Force with the LLNA has been summarised in the following report: A. Penninks (2006): Limitations of the Local Lymph Node Assay (LLNA) as preferred test for skin sensitisation: concerns about false positive and false negative test results, TNO report V7217).

CESIO would appreciate if this report were included in the ICCVAM evaluation process of the LLNA.

CESIO has encouraged its membership to submit data discussed in the Penninks (2006) review to NICEATM.

Yours sincerely,

Dr Kirill Skirda
CESIO
Senior Counsellor
Oleochemicals & Surfactants
E-mail: ksk@cefic.be
Tel. +32 2 676 7304
Fax +32 2 676 7347

(originally signed – sent electronically)

15 June 2007

Dr. William S. Stokes
NICEATM Director, NIEHS
P.O. Box 12233, MD EC-17
Research Triangle Park, NC 27709

RE: **Nomination of LLNA peer review panel members**

Dear Dr. Stokes:

On behalf of CropLife America the national trade association representing the crop protection industry, I respectfully nominate Dr. Gregory S. Ladics and Dr. Mike Woolhiser to sit on the Interagency Coordinating Committee of the Validation of Alternative Methods (ICCVAM) local lymph node assay (LLNA) peer review panel to review proposed LLNA uses and procedures.

Drs. Ladics and Woolhiser have extensive experience in toxicology, specifically in the field of immunotoxicology. These scientists bring a high degree of expertise in immunotoxicology and scientific objectivity that will contribute greatly to the charge of ICCVAM regarding its review of the LLNA.

Gregory S. Ladics, PhD
Senior Research Toxicologist
DuPont Crop Protection
1090 Elkton Road
Newark, DE 19714-0030
(302) 451-4606
Fax: (302) 366-5207
gregory.s.ladics@usa.dupont.com

Michael R. Woolhiser, PhD
Technical Leader – Immunotoxicology
The Dow Chemical Company
Building 1803
Midland, MI 48674
(989) 636-7549
Fax: (989) 638-9863
mwoolhiser@dow.com

Attached with this letter, please find a curriculum vitae and brief summary of relevant experience and qualifications for Dr. Woolhiser. Dr. Ladics' curriculum vitae and relevant experience will become available early next week and I will forward those documents to you when they are received.

Best Regards,

Mark S. Maier, PhD, DABT
Health Science Policy Leader

1156 15th Street, NW, Suite 400, Washington, DC 20005
202-296-1585

Date: Tue, 12 Jun 2007 16:48:59 +0200
Subject: Local Lymph Node Assay Data with Aqueous Products

On behalf of the European Crop Protection Association, I'm forwarding with this e-mail full reports of ECPA's 2006 study on the use of the mouse local lymph node assay with aqueous-based plant protection products (formulations). I also attach a summary of the study in the form of a poster presented at the March 2007 Society of Toxicology meeting.

This submission is in response to the NICEATM request published in the Federal Register on May 17th, 2007 specifically to address the question on "the ability of the LLNA to test mixtures and aqueous solutions"

SoT poster showing overview of the ECPA study:-

Paper by Ryan et al (2002) which includes evidence of the suitability of Pluronic L92 as a vehicle for aqueous materials in the LLNA - this was the basis of the ECPA "validation study"

Individual lab reports testing 3 positive control chemicals and 4 pesticide formulations in the LLNA with Pluronic L92 as vehicle:-

Dow

BASF

Bayer

Dupont

Syngenta (conducted at RCC, Switzerland)

We also intend to forward reports of the guinea pig studies conducted on the four plant protection products at Dow. These will follow shortly.

Please use me as your contact point for any queries or questions on our data and study. My coordinates are:-

Dr Phil Botham
Head of Human Safety (Europe)
Syngenta CTL
Alderley Park

Macclesfield
Cheshire
SK10 4TJ
UK

INTERLABORATORY LOCAL LYMPH NODE ASSAY USING CBA/J MICE TO EVALUATE DERMAL SENSITIZATION POTENTIAL OF PESTICIDE FORMULATIONS DILUTED IN PLURONIC L92 SURFACTANT AS A VEHICLE

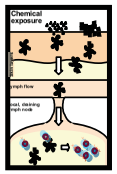
M. Woolhiser[1], C. Wiescinski[1], P. Botham[2], D. Lees[2], E. Debruyne[3], M. Repetto-Larsay[3], G. Ladics[4], D. Hoban[4], A. Gamer[5], M. Remmele[5], W. Wang-Fan[6], L. Ullmann[6], R. Billington[7] and J. Mehta[7]

The Dow Chemical Company, Toxicology and Environmental Research and Consulting, Midland, MI, USA[1], Syngenta Crop Protection, Inc., Alderley Park, UK[2], Bayer CropScience, Sophia Antipolis, France[3], The DuPont Company, Wilmington, DE, USA[4], BASF Aktiengesellschaft, Ludwigshafen, Germany[5], RCC Ltd., Itingen, Switzerland[6] and Dow AgroSciences, Oxford, UK[7]

Abstract

The mouse local lymph node assay (LLNA) had become broadly accepted and is requested as the primary animal test for evaluating the dermal sensitization potential of chemicals. As pesticide formulations are typically a specific complex blend of chemicals, traditional vehicles prescribed for the LLNA can be incompatible with these formulations leading to inaccurate test results and hazard identification. The objective of this study was to evaluate the effectiveness of an aqueous dilution of Pluronic® L92 block copolymer surfactant (L92) as a vehicle in the mouse LLNA amongst 5 labs using 3 chemicals with known sensitization potential, hexylcinnamaldehyde (HCA), 36.5% formaldehyde in water (FOR) and potassium dichromate (PDC). Identical LLNA protocols and test material lots were used. Female CBA/J mice were treated on three consecutive days with HCA (3-30%), FOR (1-20%) or PDC (0.02-0.5%) diluted in 1% L92. After 2 days rest, lymph nodes were harvested for evaluation of cell proliferation (^{3}H-thymidine incorporation). All labs observed positive LLNA responses (stimulation index (SI) >3) for the three chemicals. For HCA, the range of concentrations (6.7-17.6%) calculated to elicit 3-fold increases in proliferation (EC_3 values) were similar with that reported (8%) using acetone:olive oil. PDC elicited EC_3 values (0.06-0.33%) which encompass that reported using L92 (0.17%) or DMSO (0.06-0.18%). EC_3 values for 36.5% FOR ranged form 3.8-12.3% (v/v). These data reproduced previous results using 1% L92 and are only slightly above the EC_3 measured with FOR in acetone. LLNA results using 1% L92 as a vehicle were reproducible amongst the 5 labs and support the use of 1% L92 as a vehicle in the mouse LLNA. This work was sponsored and funded by the European Crop Protection Association.

Introduction



As certain chemicals can cause allergic reactions in humans (e.g., contact dermatitis), there remains a need for testing chemical products for the ability to cause allergy. As prediction of allergy potential can not be accurately performed using computer software or in vitro methods, the use of laboratory animals is still necessary.

Over the past several years, the mouse local lymph node assay (LLNA) has been requested by European and North American regulatory organizations as the preferred animal test to evaluate dermal sensitization potential of chemicals. However, identification of sensitization potential for individual chemical components does not necessarily define the hazard of a final product. The advantages of the mouse LLNA make it very desirable to test formulated products, and offers the additional advantage of a consistent testing paradigm between components and products.

As many product formulations (particularly pesticides) are designed using mostly aqueous ingredients, and can be incompatible with traditional LLNA vehicles (e.g., acetone), the use of standard vehicles would lead to inaccurate identification of sensitization potential of the final product.

The objective of this study was to formally evaluate the predicted accuracy of the LLNA for chemical formulations while using an aqueous dilution of Pluronic® L92 block copolymer surfactant (L92) as a vehicle in the mouse LLNA.

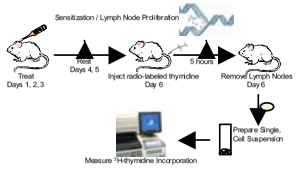
Overview of LLNA

In the LLNA, the allergy potential of a chemical (product) is measured as a function of cell proliferation in the local lymph nodes of mice exposed topically (ears) to a chemical.

To measure cell proliferation, mice are injected (tail vein) with a radio-labeled chemical (DNA nucleotide) several days after chemical applications.

A 3-fold increase in radio-nucleotide measurement between treated and untreated mice is considered to be indicative of chemical allergy potential.

The relative potency of different products can be compared by examining the chemical dose needed to cause the 3-fold increase in radio-nucleotide incorporation (i.e., EC_3 concentration).



Materials & Methods

- Female CBA mice
- 9-12 weeks of age
- 5 mice/group

Animal Welfare: In accordance with the U.S. Department of Agriculture's rules on animal welfare, 9 CFR Parts 1-4, the Animal Care and Use Activities required for the conduct of these studies were reviewed and approved by the Institutional Animal Care and Use Committee.

Test Materials

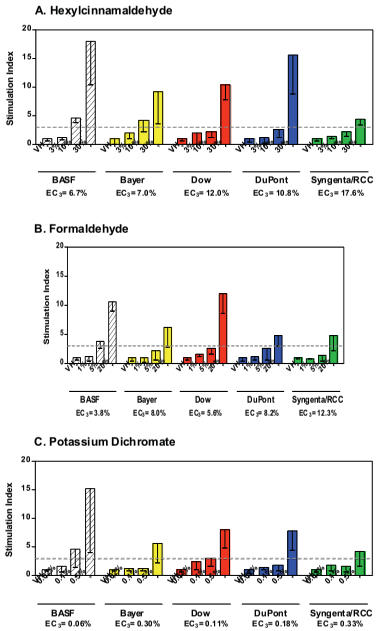
- Hexylcinnamaldehyde (HCA); Sigma-Aldrich; 95% minimum purity
- Formaldehyde (FOR); Sigma-Aldrich; 36.5-38% purity
- Potassium dichromate (PDC); Sigma-Aldrich; 99% minimum purity
- Oxyfluorfen EC; Dow AgroSciences LLC, purity not available
- Quinoxyfen/Cyproconazole SC; Dow AgroSciences LLC, purity not available
- Dinocap EC; Dow AgroSciences LLC, purity not available
- Trifluralin EC; Dow AgroSciences LLC, purity not available

(EC = emulsion concentrate; SC = suspension concentrate)

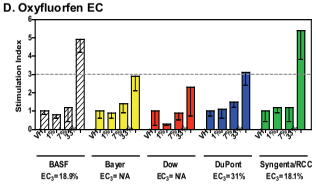
Previous Toxicity Information

[a]Known sensitizer — human

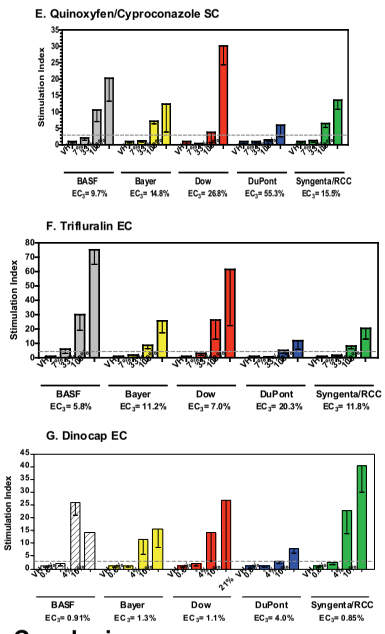
Results – Positive Controls



Results – Pesticide Products



Results – Pesticide Products



Conclusions

- **These results support the use of the mouse LLNA for testing formulated, pesticide products using 1% L92 as an alternative, aqueous-based vehicle.**
  - **Evaluation of known chemical allergens (HCA, FOR, & PDC) produced positive results using 1% L92 as a an alternative, aqueous-based vehicle. Results were reproducible among the five laboratories and demonstrated consistent, relative potencies for the three positive controls. Results indicate that a concurrent positive control is not required when using this vehicle.**
  - **Pesticide products demonstrated reproducible results using the LLNA amongst five laboratories. Results were largely consistent with that generated previously using guinea pigs or from human experience.**
- **LLNA provides both scientific and animal welfare advantages when compared to guinea pig allergy tests. *Replacement* of guinea pig models presents both a *refinement* and a *reduction* in animal usage.**

Dr. William S. Stokes
NICEATM Director
NIEHS
P.O. Box 12233
MD EC-17
Research Triangle Park
NC 27709
Via E-mail: niceatm@niehs.nih.gov

Frankfurt, June 14 2007

Dear Madam / Sir

Re. LLNA: Request for Comments, Nominations of Scientific Experts, and Submission of Data (Federal Register 72 (95), May 17, 2007, page 27815)

The European Federation for Cosmetic Ingredients (EFfCI) appreciates a.m. request of NICEATM and the opportunity to contribute with comments and available data relevant to the term of references. EFfCI member companies have experienced over the last years more and more unexpected and unexplainable positive findings in the murine local lymph node assay. Most of these materials are in consumer use for decades without exhibiting any indication of skin sensitizing properties on the basis of guinea pig tests (M+K, Buehler), human data and/or experience. Based hereupon, EFfCI installed a LLNA working group to consider the scientific accuracy of LLNA results with cosmetic raw materials. Beside mechanistic considerations also experimental work was initiated by this working group with materials which apparently are not adequately represented in the existing validation trials of the LLNA.

In this respect EFfCI sponsored the following comparative experimental test with cosmetic raw materials and which we would like to share with NICEATM. EFfCI would appreciate if this report will be included in the ICCVAM evaluation process of the LLNA:

"Comparative Experimental Study
on the Skin Sensitising Potential of
Selected Unsaturated Chemicals as
Assessed by the Murine Local Lymph Node Assay (LLNA)
and the Guinea Pig Maximisation Test (GPMT)"
(Annex)

/2

Dr. Peter Ungeheuer
Secretary General
Mainzer Landstrasse 55
D - 60329 Frankfurt

Phone : +49-69-25 56 13 41
E-mail : Ungeheuer@effci.com
Fax : +49-69-25 56 13 42
HTTP://WWW.EFFCI.COM

EFfCI Head Office: 2, Avenue de Tervueren, B-1040 Brussels • Registered as EEIG, No 480249374

In this study, eight unsaturated substances and one saturated substance - that were assumed to have low or no sensitisation potentials - were subjected to comparative testing in the LLNA and the Guinea Pig Maximisation Test (GPMT). The aim of this project was to investigate the justification or the potential limitations of the LLNA as a stand-alone method by comparing the sensitizing potential data obtained with these two different tests in strict adherence to their respective OECD guidelines.

EFfCI is also willing to actively participate in the evaluation and review process of this exercise and nominates Dr. Reinhard Kreiling, Chair of the EFfCI Toxicology Working Group as potential member of a possible peer review panel. Dr. Reinhard Kreiling is a Senior Toxicologist and Deputy Head of the Toxicology Department of Clariant GmbH, Sulzbach, Germany. A CV would be available if necessary.

We are at your disposal should you need further clarification or if you wish to discuss the results.

Yours sincerely

Peter Ungeheuer
Secretary General

Annex

On 5/21/07 2:09 PM, "Dori Germolec" <germolec@niehs.nih.gov> wrote:

I would suggest Dr. Mary Jane Selgrade at USEPA. I would suggest Dr. Jean Regal at the University of Minnesota and Dr. Michael Luster. Mike has recently retired from NIOSH and is now a consultant. I am sure that you have Drs. Kimber, Basketter and Gerberick as part of the sponsors. I would also suggest Dr. Kimber White from Virginia Commonwealth University, who is our ITOX contractor. Please let me know if you need any additional names. I am not sure if you are looking for government or extramural panelists or both.

Dori

From: Dori Germolec
Date: Fri, 29 Jun 2007 11:52:56 -0400
To: "Stokes, William (NIH/NIEHS) [E]", "Choksi, Neepa (NIH/NIEHS) [C]"
Cc: "Tice, Raymond (NIH/NIEHS) [E]"
Conversation: LLNA data for ICCVAM review

Based on the request for data from standard LLNA testing announced in the Federal Register, Thursday, May 17, 2007 (FR_E7_9544), I would like to submit 20 reports from the National Toxicology Program's effects to assess the potential for chemicals to induce hypersensitivity, which include standard LLNA testing. Because these are large files I will copy them to a CD-ROM and hand deliver the disc to the ICCVAM office. A majority of these reports also include other studies such as the Mouse Ear Swelling test. I have an additional 17 reports evaluating chemical-induced hypersensitivity that do not include the LLNA, as these studies were conducted before the development of the standard protocol. Please let me know if these reports would also be informative for your data review.

Dori Germolec
Integrative Toxicology Group
NIEHS
79 Alexander Drive
PO Box 12233
Research Triangle Park, NC 27709
T: (919) 541-3230
F: (919) 541-0870

Subject: FR Notice Comments - 72FR27815 - LLNA
Date: Friday, June 15, 2007 1:43 PM

Dear Dr Stokes,

Safepharm Laboratories Ltd., UK (SPL) has conducted Local lymph node assays on behalf of sponsoring companies since 1997. The assays have been conducted on a wide variety of chemicals and chemical preparations. Since August 2002 the use of other animal models for evaluation of skin sensitisation potential for regulatory purposes (e.g. methods that require the use of guinea pigs) has been permissible in the UK only if a valid scientific reason can be provided as to why a LLNA cannot be conducted. In effect, the LLNA is the only method that can be used in the UK for assessment of skin sensitisation potential forregulatory purposes. We therefore support the proposed activities of ICCVAM-NICEATM as detailed in the Federal Register vol. 72, No. 95, p.27815-27817, 17 May 2007 in response to the U.S. CPSC nomination of January 10, 2007.

We have witnessed concerns in some areas of the chemical industry, with regard to the applicability of the LLNA for testing of preparations, mixtures and irritant substances, and also with regard to the fact that the LLNA has not always provided results consistent with existing knowledge of the test substance or related test substances. We do not know if all of these concerns are justified, but they can only serve to reduce confidence in the predictive capability of the assay. This is not desirable when the assay offers significant scientific and animal welfare advantages over guinea pig models for many product types, and in a country where the assay is effectively the only available method for evaluation of skin sensitisation potential for regulatory purposes. An assessment of the applicability domain of the assay in its current form and the use of the assay for testing mixtures, preparations, aqueous solutions, irritant substances and metals is therefore very much welcomed. It seems very appropriate to initiate a review of the current peer-reviewed literature and available data, in order to prepare a comprehensive background review document, conduct a review of the validation status of the LLNA for its various uses and to develop relevant performance standards.

It is noted that at its 26th meeting held on 26-27th April 2007 at the European Centre for the Validation of Alternative Methods (ECVAM), the non-commission members of ECVAM Scientific and Advisory Committee (ESAC) considered the reduced version of the LLNA (rLLNA) to be scientifically validated, but only when used as a screening test to distinguish between sensitisers and non-sensitisers and with due regard to the conditions set forth in the official ESAC statement of 27th April 2007. This statement was based on the outcome of a review of LLNA data for 211 chemicals[1]. The review of existing and newly-provided LLNA data proposed by ICCVAM-NICEATM therefore presents an ideal opportunity to assess further the validity of the rLLNA for screening purposes.

As a contract research organisation, SPL is unable to provide data for review by ICCVAM-NICEATM without the permission of its Sponsors, although we

consider it may be possible to provide a summary of study outcomes, coupled to general product type, should this be of interest to ICCVAMNICEATM.

In conclusion, Safepharm Laboratories Ltd. welcomes the proposed activities of ICCVAM-NICEATM in response to the U.S. CPSC nomination of January 10, 2007, and will be pleased to explore ways in which our experience may be of use in the process.

Yours sincerely,
Robert L. Guest
Head of Alternative and Acute Toxicology
Safepharm Laboratories Ltd.

[1] I Kimber, RJ Dearman, CJ Betts, GF Gerberick, CA Ryan, PS Kern, GY Patlewicz, DA Basketter (2006). The local lymph node assay and skin sensitization: a cut-down screen to reduce animal requirements? Contact Dermatitis 2006: 54:181-185

MB Research Labs
Experience and Innovation
1972

1765 wentz road, p.o. box 178
spinnerstown, pa 18968
phone (215) 536-4110
fax (215) 536-1816
mbinfo@mbresearch.com

June 4, 2007

Dr. Mary Wolfe
Director, NTP Liaison and Scientific Review Office
NIEHS/NIH
P.O. Box 12233, MD A3-01
111 TW Alexander Drive
Research Triangle Park, NC 27709

RE: Nominations to ICCVAM, Non-Radioactive Murine Local Lymph Node Assays, Request for Comment, *Federal Register, Vol. 72, No.83, pages 23831-23832, May 17, 2007*

Dear Dr. Wolfe and Honorable Committee Members:

In response to the Consumer Product Safety Commission's request to NICEATM and ICCVAM to evaluate non-radioactive versions of the Local Lymph Node Assay (LLNA), MB Research Laboratories would like to offer its support for this nomination and extend our assistance and available information towards the validation of non-radioactive LLNA methods.

MB Research Laboratories has developed and routinely performs a commercial research protocol for the assessment of acute dermal sensitization using a **Flow Cytometry-based Local Lymph Node Assay – FC-LLNA**. In contrast to the radioactive LLNA, the FC-LLNA assesses proliferation by determining incorporation of the thymidine analog bromodeoxyuridine (BrdU) into the DNA of lymph node cells, along with evaluation of lymph node cell number, using flow cytometric methods. It is safer to conduct because of the elimination of hazardous radioactive material, and with added endpoints, is able to better identify true sensitizers and false positive irritants.

The FC-LLNA is a direct result of a three-year SBIR grant project (R44-ES-10234-02). The goal of the project was to develop a commercially viable assay that would be a significant improvement over the standard radioactive LLNA while maintaining high levels of accuracy, sensitivity, specificity, and predictivity. During the conduct of our internal validation studies, over 50 chemicals, including sensitizers, nonsensitizers and irritants were tested. Since 2001, more than 80 FC-LLNA studies have been conducted by clients in the chemical, pharmaceutical, and consumer product industries for safety evaluations and potential submission to regulatory agencies.

The FC-LLNA is very similar to the ICCVAM-validated LLNA protocol but adapted for flow cytometric evaluation. Specifically, the dosing method, assay schedule, vehicles and positive controls are identical. Of the similarities, most noteablely both assays evaluate lymphocyte proliferation and designate a cut off value of stimulation index (SI) = 3 as a positive indication of sensitization.

The significant difference between the two protocols is that in the radioactive LLNA mice are injected by tail vein with ^{3}H thymide, while in the FC-LLNA mice are injected intraperitonially with BrdU. Additionally, because the cells are not radiaoactively labeled, an aliquot can also be stained for immunophenotyping and activation marker analysis, thus reducing the need for additional animal groups. Profiling of immunophenotypic markers such as B220, CD3, I-A^{k} and CD69 can be added to our basic protocol to distinguish between sensitizers and false positive irritants. Ear swelling measurements have also been included to the basic FC-LLNA test to evaluate irritation of test articles and screen for possible false positives.

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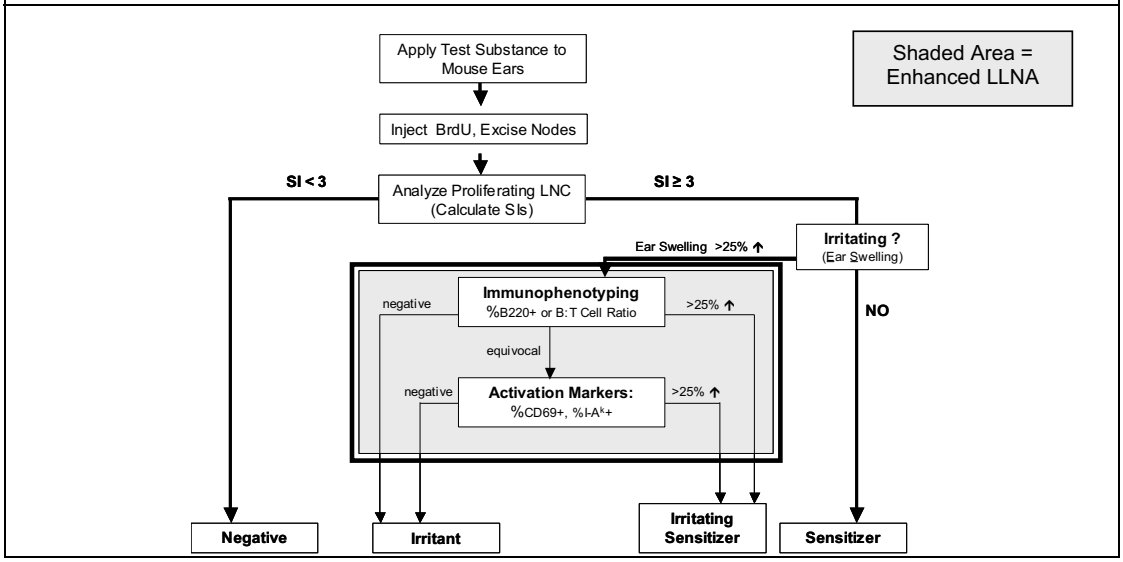
In the FC-LLNA, proliferation of lymph node cells is measured by a combination of BrdU incorporation and total lymph node cell number. As with the radioactive version of the LLNA, an SI of 3 or greater indicates a positive sensitizing response. Each treatment group consists of five mice. Each mouse is evaluated independently by multiplying the total number of lymphocytes by the percentage of lymph node cells that are positive for BrdU incorporation. The total number of proliferating cells in the test group is divided by the total number of proliferating cells in the vehicle group to give a stimulation index. The FC-LLNA yields SI's similar to those in the ICCVAM validation report as well as other published results for the radioactive LLNA. The estimated concentration of chemical required to induce an SI of 3 (EC3), can be used to determine the potency of sensitizers. EC3 values obtained in the cytometric LLNA are quite comparable to those found in the radioactive LLNA, and in most cases fall within the range of values obtained for chemicals tested in the radioactive assay. (See Table 1)

For our validation, immunophenotype analysis of the nodal cells was conducted using the marker combinations B220/CD3 to determine the ratio of B cells to T cells and I-A^k/CD69 to determine the activation state of the nodal lymphocytes. More specifically, to investigate activation state, the murine MHC class II alloantigen (IA) surface marker was evaluated and the percentage of the total nodal percentage of I-A^k+ cells that were also positive for the CD69 marker was determined. A major advantage of the FC-LLNA is that immunophenotype analysis can be performed on an aliquot of the cells harvested for SI analysis and no additional animals need be used.

An illustration of the FC-LLNA multi-tiered approach to evaluate sensitizers and eliminate false-positive irritants is shown in Figure 1.

Figure 1. Multi-tiered Testing Strategy for the Assessment of Sensitization Potential using the FC-LLNA.



In the first tier, an SI<3 indicates a non sensitizer. For chemicals that elicit an SI>3, ear thickness measurements can be utilized as an indication of irritancy, since CBA mice are brown, thus erythema cannot be evaluated. In the second tier of our FC-LLNA, positive ear swelling flags possible false positive irritants due to the fact that irritants dramatically increase the thickness of the ear, while contact allergens induce a minimal increase in skin thickness due to low inflammatory response. In the last tier, immunophenotyping markers are used to distinguish between true sensitizers and false positive irritants. These markers strongly correlate to positive sentization potential. Additionally, we have found that some irritants do not increase ear swelling, but can be distinguished from sensitizers because of a lack of immunophentypic response.

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Table 1 is a list of compounds tested in the FC-LLNA compared to the radioactive LLNA based on SI alone. Also included in the table are a group of equivocal compounds, which were not included in contingency table evaluations.

Table 1: LLNA Compound List Comparing MB Research Flow Cytometry (FC) LLNA Results with ICCVAM Validation Radioactive (R) LLNA Results

Positive by Radioactive LLNA	FC	R
2,4-dinitrochlorobenzene	+	+
Aminophenol HCL	+	+
Benzoyl peroxide	+	+
Chlorpromazine +UVR	+	+
Citral	+	+
Cobalt chloride	+	+
Copper chloride	+	+
Croton Oil	+	+
Diethylenetriamine	+	+
Diphenylcyclopropenone	+	+*
Ethylene glycol dimethacrylate#	+	+
Eugenol	+	+
Fluorescein isothiocyanate	+	+
Formaldehyde	+	+
Hexylcinnamaldehyde	+	+
IsoEugenol	+	+
Isopropyl Myristate	+	+*
Linalool	+	+*
Oxazolone	+	+
Potassium dichromate	+	+
p-phenylenediamine	+	+
Pyridine	+	+
Sodium lauryl sulfate#	+	+
Tetrachlorosalicylanilide	+	+
Trimellitic anhydride	+	+*
Xylene	+	+

Negative by Radioactive LLNA	FC	R
6-methyl coumarin	–	–
Benzoic acid	–	–
Chlorobenzene	–	–
Glycerol	–	–
Hexane	–	–
Hydrocortisone	–	–
Isopropanol	–	–
Lactic acid	–	–
Methyl salicylate	–	–
Nickel chloride	–	–
p-aminobenzoic acid	–	–
Propylene glycol	–	–
Propylparaben	–	–
Resorcinol	+	–
Sulfanilamide	–	–
Tween 80	+	–

Equivocal	FC	R
Aniline	–	+/–
Benzalkonium chloride#	+	+/–
Benzocaine	+/–	+/–
Ethylenediamine	+	+/–
MBT	+/–	+
Salicylic acid	+/–	–

* = HSE contract research report 399, 2001. Development of the Local Lymph Node Assay for Risk Assessment of Chemicals and Formulations, Rebecca J. Dearman and Ian Kimber, Syngenta Central Toxicology Laboratory, UK, 2001, p.12.
# = Classify as irritants but not sensitizers using the enhanced FC-LLNA with immunophenotype endpoints.

We have also provided in Table 2, a comparative evaluation of data from the flow cytometric assay (FC), the radioactive assay (R), guinea pig results (GP) and human data (H). The cytometric assay has 95% accuracy to the radioactive assay, as well as 93% sensitivity and 100% specificity. Moreover, while the FC-LLNA is less accurate than the radioactive assay when compared to the guinea pig assay (79% vs. 89%) it is more accurate than the radioactive test when compared to human data (88% vs. 72%).

Table 2: Comparative Evaluation of the Flow Cytometric LLNA

Comparison of Method	Total #	Accuracy		Sensitivity		Specificity		Positive Predictivity		Negative Predictivity	
		%	#	%	#	%	#	%	#	%	#
FC-LLNA vs. R-LLNA	42	**95%**	40/42	**93%**	26/28	**100%**	14/14	**100%**	26/26	**88%**	14/16
FC-LLNA vs. Human	26	88%	22/25	90%	18/20	83%	5/6	95%	18/19	71%	5/7
R-LLNA vs. Human	74	72%	53/74	72%	49/68	67%	4/6	96%	49/51	17%	4/23
FC-LLNA vs. Guinea Pig*	29	79%	23/29	74%	14/19	90%	9/10	93%	14/15	64%	9/14
R-LLNA vs. Guinea Pig*	97	89%	86/97	91%	62/68	83%	24/29	93%	62/67	80%	24/30

Radioactive LLNA results obtained from ICCVAM Validation of the LLNA[b]
* = Results from Guinea Pig Maximization Test and/or Beuhler Assay

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Augmentation of the original LLNA with the flow cytometry endpoints increases the sensitivity and discriminating power of the LLNA, while (1) complying with the Animal Welfare Act by directly addressing the reduction in animal number; (2) increasing the quality and quantity of data generated when compared to existing methods; and (3) substantially reducing the cost of analysis and waste disposal by avoiding the use of radioactivity.

In conclusion, MB Research Laboratories fully supports the Consumer Product Safety Commission's nomination to ICCVAM for the evaluation of the non-radioactive LLNA methods for classifying sensitizers and offers to assist ICCVAM by offering the FC-LLNA protocol, validation data and methods for consideration as a direct substitute to the Guinea Pig Sensitization Test.

/s/

Daniel R. Cerven, MS
Director of Laboratories
MB Research Laboratories

/s/

Melissa K. Kirk, Ph.D.
Study Director/Lab Supervisor
MB Research Laboratories

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June 14, 2007

Dr. William S. Stokes
Director, NICEATM and
Executive Director, ICCVAM
National Institute of Environmental Health Sciences
PO Box 12233, MD EC-17
Research Triangle Park, NC 27709

Re: FR notice dated May 17, 2007 (CPSC nomination of Local Lymph Node Assay) - response to call for nominations for potential Expert Panel [72 FR 23832].

Dear Dr. Stokes,
This letter is in response to the request for comments on the US CPSC proposal to ICCVAM-NICEATM for an updated evaluation of the validation status of the murine local lymph node assay. I am pleased to submit the nomination of Dr. G. Frank Gerberick to serve on the proposed expert panel to review an updated LLNA Background Review Document and (a.) the validity of proposed modifications to the LLNA (eg. non-radioactive protocols), (b.) as a stand-alone assay for potency determination for classification purposes, and (c.) to explore applicability domains to address Regulatory concerns over the LLNA's validity for testing mixtures, aqueous solutions, and metals. It is expected that this Expert Panel would also review any proposed ICCVAM recommendations for: (e.) current uses and/or limitations for above methods, (f.) test method protocols and/or decision criteria, (g.) performance standards, and (h.) future/additional studies.

Dr. Gerberick is an esteemed colleague at the Procter & Gamble Company in our corporate research division overseeing the global Skin Irritation/Contact Sensitization program. His extensive work over the past two decades in the field of dermal irritation/contact sensitization has made him one of the world's foremost authorities on contact allergy and dermal sensitization. This work has included his pioneering work with the LLNA assay and *in vitro* and *in silico* test methodologies for better scientific understanding of the risk factors for dermal sensitization. It is my opinion that he would be a substantial asset to ICCVAM/NICEATM in the evaluation of new information and proposed applications for the assay he helped pioneer.

Thank you for your thoughtful consideration,

Dan

Daniel S. Marsman, DVM, PhD
Section Head, Animal Welfare and Alternatives
BB-1S479B WHBC
Procter & Gamble
6280 Center Hill Drive,
Cincinnati, OH 45224
Ph: (513) 698-6088
Fax: (886) 357-7849
marsman.ds@pg.com

Date: Thu, 7 Jun 2007 09:00:44 -0400
Subject: NTP NICEATM Nomination of experts and response to call for data - LLNA
Ref.: Federal Register vol. 72 no. 95, p. 27815, 17 May 2007

Dr. Stokes -
Responding to the request for comment on the US CPSC proposal to ICCVAM-NICEATM for evaluation of the validation status of the murine local lymph node assay, I am pleased to submit the following information for consideration. (The views expressed in item 1.) below are solely my own and do not necessarily reflect the corporate position of GSK.)

1.) Appropriateness and relative priority of items comprising the proposed review of the status of the LLNA: It seems entirely justified that the proposed review should be undertaken based on the large volume of high quality peer-reviewed information published on performance, data evaluation and proposed protocol modifications of the LLNA in the period since the original ICCVAM-sponsored LLNA validation exercise. As proposed by US CPSC, ICCVAM-NICEATM preparation of a comprehensive background review should precede activation of a study panel. Regarding the priority of items for the background review as presented in the Federal Register notice, I suggest that the priority sequence should be slightly rearranged to highlight items 1, 5, 4, 2 and 3 (as identified in the Fed. Reg. notice) in priority sequence. Thus, from most to least pressing: 1. development of data to allow the LLNA to be used as a stand-alone tool in determining potency / severity of sensitising potential of chemicals; 2. evaluation and extension of the domain of applicability of the LLNA; 3. use of the LLNA for testing mixtures, aqueous solutions, and metals; 4. development of an animal-sparing cut-down approach to the LLNA focused on use of untreated vs. single high-concentration test group; and 5. assessment of the status of LLNA methods using non-radiolabeled tracer for end-point analysis.

2.) Nomination of expert scientists to serve on a possible LLNA review panel: I am pleased to offer the name of my GSK colleague Frederick J. Guerriero as a possible panel member. Mr Guerriero is a key member of the GSK Occupational Toxicology working group and in this capacity has had the responsibility of protocol development, study contracting and evaluation of a large number of LLN assays over the past 7-8 years. In addition, Mr Guerriero has previously served on the NICEATM study panel which evaluated *in vitro* alternatives for evaluation of ocular irritant/corrosion effects of chemicals. As a secondary potential candidate for the study panel, I would also be pleased to volunteer my service which is based in similar experience to that of Mr. Guerriero.

3.) Submission of LLNA data: Over the past 5 years GSK has transitioned to sole use of the LLNA as a means for evaluating the sensitising potential of a wide variety of chemical materials used in the synthesis of pharmaceuticals. The spectrum of substances which have been evaluated includes commodity chemicals used as starting materials, proprietary synthetic intermediates of varying structural complexity, and active pharmaceutical entities. All of these

assessments have been conducted by the "traditional" control + 3 concentration protocol using 3H-thymidine label. A small proportion of materials also have companion data evolved with the M&K or Beuhler dermal sensitisation protocol. Although the composite data are not presently in a readily transmitted form, I believe that we could be in position to share results of assessment of *ca.*190 chemicals if materials from the pharmaceutical sector would be of interest in the assessment which NICEATM is planning.

I will send this letter in print form with mailing today. I look forward to your reply in due course.

Sincerely yours -
Michael J. Olson, Ph.D.
Director, Occupational Toxicology
Corporate Environment, Health and Safety
GlaxoSmithKline

Research Institute for Fragrance Materials, Inc.
50 Tice Boulevard
Woodcliff Lake, New Jersey 07677 USA
Phone: 201-689-8089 FAX: 201-689-8090

June 15, 2007

Dr. William S. Stokes
NICEATM Director
NIEHS
P.O. Box 12233
MD EC-17
Research Triangle Park, NC 27709

Dear Dr. Stokes:

This letter is in response to the NICEATM request for data on the murine local lymph node assay that appeared in the Federal Register on Thursday May 17, 2007 (Volume 72, No. 95, p. 27815).

The Research Institute for Fragrance Materials, Inc. (RIFM), the international scientific authority for the safe use of fragrance materials, is the most comprehensive source of toxicology data, literature and information on the safety evaluation of fragrance materials. Through extensive research and testing and constant monitoring of all scientific literature available, RIFM maintains a database of fragrance and flavor materials considered the largest repository of this type of information in the world. All of RIFM's scientific findings are evaluated by an independent, scientific Expert Panel—an international group of dermatologists, pathologists, toxicologists and environmental scientists who are completely unbiased with no connection to the fragrance industry. More information about RIFM can be found on the RIFM web site at www.rifm.org.

The murine Local Lymph Node Assay (LLNA) has provided toxicologists with a tool that provides both a reduction in the use of animals and a refinement over traditional assays for hazard identification and potency classification of contact sensitizers. Since 2000, RIFM has used the LLNA almost exclusively for this purpose. The data that RIFM has generated in the LLNA has been incorporated into several publications that aim to provide a standardized data set for the development of alternative methodologies.

RIFM has explored the use of the LLNA in various essential oils. Mr. Jon Lalko, RIFM Senior Test Program Specialist managed this project, which had two goals: 1) to investigate the potential of individual essential oils to induce dermal sensitization and to determine the relative potency of the oil; and 2) to examine any difference in sensitization potential for the major components arising form their exposure. The initial work was published in *Food and Chemical Toxicology* (2007), Volume 44, pp. 739-746). A copy of the publication is attached. RIFM has continued to investigate the use of the LLNA in various essential oils. Enclosed is a summary of the LLNA data RIFM has sponsored on several essential oils.

Much work has been done to correlate the dose-response data obtained in the mouse LLNA with what is known about potency in humans. The EC3 value has recently been demonstrated to closely correlate with the NOEL from human sensitization tests designed to confirm lack of induction. RIFM has compared the relationship between the LLNA EC3 value and the NOEL for sensitization in humans. A detailed analysis of the dermal sensitization data for 31 fragrance ingredients that have exhibited dermal sensitization potential revealed that for the majority of the materials, there is a very good correlation between the EC3 or predicted NOEL from the LLNA and the NOEL in confirmatory human tests. This preliminary analysis was presented at the World Health Organization/International Program On Chemical Safety International Workshop On Skin Sensitization In Chemical Risk Assessment last October. The abstract, which is in press, is attached.

We hope that these data are useful. If there is any more information or details that we can provide, please feel free to contact me.

Best regards.

Sincerely,

Anne Marie Api

Anne Marie Api, Ph.D.
Vice President,
Human Health Sciences

AMA/caj

cc: Jon F. Lalko
Ladd W. Smith

From: "Thorne, Peter S"
Date: Mon, 21 May 2007 06:50:09 -0500
To: Neepa Choksi
Subject: RE: ICCVAM/NICEATM FR Notice: LLNA Nomination and Request for Data

Dear Dr. Choksi:

As you may know, I served on the panel that reviewed the LLNA as the first ICCVAM method. At that time the process was new and less developed than now. One of the challenges we faced was comparing somewhat limited data that were derived from non-uniform methodology. It certainly seems appropriate to take another look at the LLNA at this time and to develop performance standards. I suspect that a new data set will be richer, more methodologically uniform, and likely will include a wider range of compounds for consideration. Thus, it is an appropriate activity and deserves this further attention.

Sincerely,

Peter S. Thorne, PhD
Professor and Director
The University of Iowa
Environmental Health Sciences Research Center

June 15, 2007

Dr William S Stokes
Director, NICEATM
National Institute of Environmental Health Sciences
PO Box 12233, MD EC-17
Research Triangle Park, NC 27709

Re: **72 FR 27815; May 17, 2007; National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); the Murine Local Lymph Node Assay (LLNA): Request for Comments, Nominations of Scientific Experts, and Submission of Data**

Dear Dr. Stokes:

These comments are submitted on behalf of the Alternatives Research and Development Foundation, the American Anti-Vivisection Society, Humane Society Legislative Fund, The Humane Society of the United States, People for the Ethical Treatment of Animals and the Physicians Committee for Responsible Medicine. The parties to this submission are national animal protection, health, and scientific advocacy organizations with a combined constituency of more than 10 million Americans who share the common goal of promoting reliable and relevant regulatory testing methods and strategies that protect human health and the environment while reducing, and ultimately eliminating, the use of animals.

In January, 2007, (ICCVAM) received a nomination from the U.S. Consumer Product Safety Commission (CPSC) to evaluate the validation status of: (1) The murine local lymph node assay (LLNA) as a stand-alone assay for determining potency (including severity) for the purpose of hazard classification; (2) the ''cut-down'' or ''limit dose'' LLNA approach; (3) non-radiolabeled LLNA methods; (4) the use of the LLNA for testing mixtures, aqueous solutions, and metals; and (5) the current applicability domain (i.e., the types of chemicals and substances for which the LLNA has been validated).

ICCVAM reviewed the nomination, assigned it a high priority, and proposed that NICEATM and ICCVAM carry out the following activities in its evaluation: (1) Initiate a review of the current literature and available data, including the preparation of a comprehensive background review document, and (2) convene a peer review panel to review the various proposed LLNA uses and procedures for which sufficient data and information are available to adequately assess their validation status. ICCVAM also recommends development of performance standards for the LLNA. At this time, NICEATM requests: (1) Public comments on the appropriateness and relative priority of these activities, (2) nominations of expert scientists to consider as members of a possible peer review panel, and (3) submission of data for the LLNA and/or modified versions of the LLNA.

At the meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) on June 12, 2007, several comments were made that suggested ICCVAM was assuming a relatively rapid review of these issues. However, this is not borne out by the CSPC

nomination which does not mention an expedited process. In addition, ICCVAM has recommended the creation of a background review document (BRD) and review by an expert peer review panel, with no mention of an expedited process. The cost/benefit of this LLNA review has not been evaluated, and SACATM was asked to vote to accept or reject NICEATM/ICCVAM's decision to proceed without offering any alternatives. Doubts about the cost/benefit of this project caused one SACATM member to vote against proceeding.

Despite the fact that ICCVAM documents, including the Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods,[1] mention the possibility of an expedited review process, it would appear that this process has only been used in one case. Despite repeated critiques of ICCVAM for failing to act expeditiously, we are still unable to locate a description of the expedited review process in ICCVAM literature and the parameters for applying it.

In light of the fact that the LLNA has been used by regulatory agencies for classifying skin sensitizers for years and both research data and regulatory use of the LLNA have been extensively reviewed in the literature, yet another review of this widely accepted method is unwarranted. The only circumstance under which this proposal is acceptable is if ICCVAM quickly reviews the existing literature and makes an expedited evaluation regarding the relevance of this information to Agency regulatory needs. ICCVAM's limited resources should be spent validating and promoting for regulatory acceptance any of the number of non-animal methods for skin sensitization that are currently in development.

In March 1999, ICCVAM published a final peer review report concluding that the LLNA is a valid alternative to currently accepted guinea pig test methods.[2] The U.S. EPA, FDA, and OSHA announced their acceptance of the LLNA as an alternative to the guinea pig maximization test for assessing allergic contact dermatitis in October 1999. That same year, ESAC, the Scientific Advisory Committee of the European Centre for the Validation of alternative Methods (ECVAM), also endorsed the LLNA for regulatory use.

In September 2000, the European Centre for Ecotoxicology and Toxicity of Chemicals (ECETOC) published a comprehensive review of sensitization test methods with respect to hazard identification and labeling, (and?) to determine whether the various methods are appropriate for determining relative potency and risk assessment.[3] The conclusions from this review included: (1) the LLNA is a viable and complete alternative to traditional guinea pig test methods for the purposes of skin sensitization hazard identification, and (2) the LLNA is suitable for the determination of relative skin sensitizing potency and the adaptation of this method for derivation of comparative criteria such as EC3 values provides an effective and quantitative basis for such measurements. This report further recommends that "the LLNA is the recommended method for new assessments of relative potency and/or for the investigation of the influence of vehicle or formulation on skin sensitizing potency."

[1] http://iccvam.niehs.nih.gov/SuppDocs/SubGuidelines/SD_subg034508.pdf
[2] http://iccvam niehs.nih.gov/methods/immunotox/immunotox.htm
[3] ECETOC. 2000. Skin Sensitization Testing for the Purpose of Hazard Identification and Risk Assessment.

More recent work has further verified the use of the LLNA as a stand-alone method for estimating potency for regulatory purposes, including a 2005 study that concludes that there is a "clear linear relationship between LLNA-derived EC3 values and historical human skin patch data."[4] A 2007 review concludes that "The LLNA, when conducted according to published guidelines, provides a robust method for skin sensitization testing that not only provides reliable hazard identification in formation but also data necessary for effective risk assessment and risk management." In addition, a retrospective analysis of the regulatory use of the LLNA in the EU was published in 2006 and concluded that "the LLNA is satisfactory for routine regulatory use."[5] We acknowledge that the LLNA must be validated for determining sensitization potency for regulatory use; however, we urge ICCVAM to take an abbreviated test validation approach, as was recommended by the recent International Programme on Chemical Safety Workshop on Skin Sensitization in Chemical Risk Assessment:[6] "An abbreviated test validation approach may be appropriate to assess the validity of potency assessment based on the LLNA and its appropriateness for predicting sensitizing induction potency in humans."

The ''cut-down'' or ''limit dose'' LLNA approach (reduced, or rLLNA) has recently been reviewed by an ECVAM peer review panel. In April, 2007, ESAC issued a statement supporting the use of the rLLNA "within tiered-testing strategies to reliably distinguish between chemicals that are skin sensitizers and non-sensitizers "thereby reducing animal use by as much as 50%."[7] The statement also notes the following limitations: that "the test results provided by the rLLNA do not allow the determination of the potency of a sensitising chemical," and that "negative test results associated with testing using concentrations of less than 10% should undergo further evaluation"

The applicability and limitations of this modification of the LLNA have been clearly established. Therefore, in lieu of a lengthy review of this method, ICCVAM should expeditiously review and endorse the ESAC peer review and circulate harmonized testing recommendations regarding this assay to US agencies before year's-end and NICEATM should collaborate with ECVAM to address the question of concentration threshold.

Other recent work has included the development of several applications of non-radioactive detection methods for the LLNA, including BrdU incorporation, methods measuring the release of various cytokines, and methods using fluorescent markers and quantification by flow cytometry. In many cases, these methods have been shown to be as sensitive as protocols involving radio-labeling.[8] In addition, in NIH-sponsored and contract work, MB Research has shown that "for a large range of chemicals, the FC-LLNA EC3 values were consistent with

[4] Basketter et al. Predictive identification of human skin sensitization thresholds. Contact Dermatitis. 2005; 53 (5): 260-267.
[5] Cockshott et al., The local lymph node assay in practice: a current regulatory perspective. Hum Exp Toxicol 2006; 25 (7): 387-394.
[6] http://www.who.int/ipcs/methods/harmonization/areas/sensitization_summary.pdf
[7] http://ecvam.jrc.it/publication/ESAC26_statement_rLLNA_20070525-1.pdf
[8] Takeyoshi et al. Advantage of using CBA/N strain mice in a non-radioisotopic modification of the local lymph node assay. J Appl Toxicol. 2006. 26:5-9. Takeyoshi et al. Novel approach for classifying chemicals according to skin sensitizing potency by non-radioisotopic modification of the local lymph node assay. J Appl Toxicol. 2005. 25:120-134. Suda et al. Local lymph node assay with non-radioisotope alternative endpoints. J Toxicol Sci. 2002. 27:205-218.

those reported in ICCVAM LLNA validation studies."[9] Both ECVAM and Japanese Center for the Validation of Alternative Methods (JaCVAM) are currently reviewing these methods and, rather than initiate a full independent review, ICCVAM must collaborate with these ongoing efforts.

With regard to the assessment of the LLNA for aqueous mixtures and metals, the information that is currently available should allow ICCVAM to make a rapid determination of the applicability and limitations of the LLNA for these classes of chemicals and, if it cannot, we do not endorse further validation efforts in this regard, but recommend the pursuit of *in vitro* methods for this purpose.

Several non-animal methods for estimating sensitivity are under development, including quantitative structure activity relationship (QSAR) modeling that shows a high concordance with both guinea pig and LLNA data,[10] quantification of peptide reactivity, which also shows a high concordance with LLNA data,[11] and human cell cultures.[12] We urge ICCVAM to secure an interagency grant from the CPSC to fund the validation of one or more of these non-animal methods. Clearly, ICCVAM and the CPSC both benefit from the sharing of resources, as the CPSC nominated the method and ICCVAM will be tasked with the final work product.

ICCVAM should consider taking an approach similar to the European Sens-it-iv project,[13] which involves the coordinated efforts of more than two dozen groups from industry, academia and other organizations, all working toward the common goal of developing *in vitro* methods to assess immunotoxicity. ICCVAM should consider facilitating the creation of such a goal-oriented task force.

To summarize, given the fact that the LLNA has been used by regulatory agencies for classifying skin sensitizers for years and both research data and regulatory use of the LLNA have been extensively reviewed in the literature and by other countries, yet another lengthy review of this widely accepted method is clearly unwarranted. Instead, we urge ICCVAM to perform an expedited review of the existing information regarding the LLNA's performance and limitations and to issue recommendations to US agencies with all due speed. In the interest of eventual replacement of animals in sensitization testing, ICCVAM must spend its time and resources promoting the development and regulatory use of non-animal methods, which it can do by engaging in integrated approaches to *in vitro* immunotoxicity.

Sincerely,

[9] http://www.mbresearch.com/TOXNOTE/TOXNOTE-LLNA.pdf
[10] Fedorowicz et al., Structure-activity models for contact sensitization. Chem Res Toxicol. 2005; 18(6): 954-969.
[11] Gerberick et al. Quantification of chemical peptide reactivity for screening contact allergens: a classification tree model approach. 2007; 97(2): 417-427.
[12] Schoeters et al. Microarray analyses in dendritic cells reveal potential biomarkers for chemical-induced shin sensitization. 2007; 44(12): 3222-3233.
[13] http://www.sens-it-iv.eu/

/s/

Catherine Willett, PhD
Science Policy Advisor
Regulatory Testing Division
People for the Ethical Treatment of Animals

/s/

Sara Amundson
Executive Director
Humane Society Legislative Fund

/s/

Dr. Martin Stephens
Vice President for Animal Research Issues
Humane Society of the United States

/s/

Kristie Stoick, MPH
Research Analyst
Physicians Committee for Responsible Medicine

/s/

Sue A. Leary
President
Alternatives Research & Development Foundation

/s/

Tracie Letterman, Esq.
Executive Director
American Anti-Vivisection Society

Subject: LLNA evaluation
Date: Wednesday, October 24, 2007 3:38 AM
From: Ann-Therese Karlberg

Dear Dr Allen,
Since my group is one of the groups in academia that performs the Local lymph node assay most frequently (one a week for many years) as part of our research program I received your mail from a Danish college.
The thing that I want to comment on is the lack of thorough chemical considerations in the choice of the substances used for testing. The substances chosen for testing should be pure, with conclusive structures and no mixtures in different ways. I will give you two examples among the substances discussed in the lists: 1. Abietic acid is considered a moderate sensitizer. In our investigations of abietic acid we found it extremely easily oxidized when exposed to oxygen in air. Abietic acid itself is not an allergen but is activated by air exposure on normal storage and handling so that allergenic oxidation products are formed in a complex mixture. The most prominent allergens identified are the hydroperoxides which as such also are unstable. In fact it is not possible to keep abietic acid pure and non-oxidized unless it is stored under argon. This makes abietic acid an unsuitable compound for evaluation of LLNA since the activity can vary depending on storage conditions of the substance. 2. Citral consists of the two stereoisomers geranial and neral which are both moderate allergens according to LLNA in our hands. Whether the results obtained in the tests with citral are due to reactions to geranial or neral or both have never been discussed. What can be said is that the dose estimated is not conclusive. Since both geranial and neral are available on the market there is no need to test them in a mixture and get non-conclusive results.
Furthermore, I think it is important that substances with an allergenic activity based on different types of reactive sites should be included to eliminate that only certain types of reactive chemicals are tested. If there are thing that you want to discuss more in detail I would be happy to discuss with you.

Best regards,

Ann-Therese Karlberg
Professor
Dermatochemistry and Skin Allergy
Department of Chemistry
Göteborg University
SE-412 96 Gothenburg
Sweden

Subject: FR Notice Comments - 72FR52130: LLNA Performance Standards
Date: Monday, September 24, 2007 11:39 AM

Below is the result of your feedback form. It was submitted by
() on Monday, September 24, 2007 at 11:39:19

Comment_date: 24 September 2007

Prefix: Dr.

FirstName: Jon

LastName: Richmond

Degree: BSc MB ChB FRDSEd FRMS

onBehalfOf: no

Title:

Department:

Company:

Country: UK

Phone:

EMail:

Comments: This is welcome development, the general approach seems sound, and I have only a few constructive comments at this stage.

1. The document does not set out the need for or divers of a need to develop and validate alternative indices of lymphocyte proliferation.

2. At 2.3.2.2, after line 419, details of all audits and copies of all audit reports should be included.

3. Appendix A, animal selection and preparation, animal species selection: lines 633/634: ths gender and strain are separate consideration and should be listed as separate bullet points.

4. Annex A, animal preparation, line 647 - the acclimitization period whould be AT LEAST 5 days. Also it is not clear if ear-punching or -notching or ear-clips are acceptable means of marking/identifying animals.

5. Annex A, selection of doses, line 699. Whilst it is reasonably clear to those familair with the key reference documents what is in tended, strictly speaking in plain English consecutive doses wold by 100%, 99%, 98% etc.

Subject: FW: ICCVAM/NICEATM FR Notice related to the murine LLNA
Date: Monday, October 22, 2007 11:34 AM
From: Henk van Loveren <Henk.van.Loveren@rivm.nl>

Dear dr. Allen

Thank you for giving us the opportunity to respond to the draft ICCVAM performance standards for the murine LLNA: methods for assessing lymphocyte proliferation.

We have discussed the draft in my group (Janine Ezendam, Rob vandebriel, Wim de Jong) and have the following comments:

Add to line 316 after LLNA: "Especially for the latter category of products to be investigated adaptations may be possible to overcome this problem. See ASTM protocol F2148-01.

ASTM F2148-01. Standard practice for evaluation of delayed contact hypersensitivity using the murine local lymph node assay. ASTM F2148-01, West Conshohocken, PA, USA.

Add to line 337 after proliferation: Should perhaps possible other endpoints be mentioned here? In any case, also modifications in determination of cellular proliferation exist that use ex vivo DNA labeling with tritium-thymidine and should be mentioned here (Kimber and Weisberger 1989, Van Och et al 2000).

Kimber, I., Weisenberger, C. A murine local lymph node assay for the identification of contact allergens. Assay development and results of an initial validation study. Arch. Toxicol. 63, 274?282, 1989.

Van Och, F.M.M., Slob, W., De Jong, W.H., Vandebriel, R.J., Van Loveren, H. A quantitative method for assessing the sensitizing potency of low molecular weight chemicals
using a local lymph node assay: employment of a regression method that includes determination of the uncertainty margins. Toxicology 146, 49?59, 2000.

Add to note 5 at page 6: An alternative mice strain that is frequently used is the BALB/c strain which shows similar responses as the CBA mice (Woolhiser et al 2000).

Woolhiser MR, Munson AE, Meade BJ. Comparison of mouse strains using

the local lymph node assay. Toxicology 146, 221-227, 2000.

Line 441: Delete 20. This gives the impression that you need to validate each alternative assay with these 20 compounds. Or is this the intention?

Line 532:
Why is the CV limited to 30% ? This looks reasonable but in table 2-3 for DNCB two out of 6 laboratories have a CV above 30%, of 35 and 46% respectively.

Line 636: A comparison of the performance of several mouse strains in the LLNA is presented in Woolhiser et al 2000.

Line 659: An example is presented in ASTM protocol F2148-01.

Line 717: The pooling approach should be discouraged as a statistic evaluation is not possible and non responding outliers cannot be detected. Also in the ICCVAM evaluation and proposed protocol pooling is not recommended. Include in text preference for individual sampling an d determination of cell proliferation.

Line 743: Add text: For this reason individual sampling should be recommended.

****

Prof. dr. Henk Van Loveren
National Institute of Public Health and the Environment
PO Box 1
3720 BA Bilthoven
the Netherlands
tel.....+31(0)302742476
mobile.... +31(0)646166122
fax.....+31(0)302744437

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Subject: FR Notice Comments - 72FR52130: LLNA Performance Standards
Date: Monday, October 29, 2007 4:31 PM

Dr William S Stokes
Director, NICEATM
National Institute of Environmental Health Sciences
PO Box 12233, MD EC-17
Research Triangle Park, NC 27709

Re: 72 FR 52130; September 12, 2007; National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments.

Dear Dr. Stokes:

These comments are submitted on behalf of the Alternatives Research and Development Foundation, the American Anti- Vivisection Society, Humane Society Legislative Fund, The Humane Society of the United States, People for the Ethical Treatment of Animals, and the Physicians Committee for Responsible Medicine. The parties to this submission are national animal protection, health, and scientific advocacy organizations with a combined constituency of more than 10 million Americans who share the common goal of promoting reliable and relevant regulatory testing methods and strategies that protect human health and the environment while reducing, and ultimately eliminating, the use of animals.

In January, 2007, (ICCVAM) received a nomination from the U.S. Consumer Product Safety Commission (CPSC) to evaluate the validation status of: (1) The murine local lymph node assay (LLNA) as a stand-alone assay for determining potency (including severity) for the purpose of hazard classification; (2) the "cut-down"˙ or "limit dose" LLNA approach; (3) non-radiolabeled LLNA methods; (4) the use of the LLNA for testing mixtures, aqueous solutions, and metals; and (5) the current applicability domain (i.e., the types of chemicals and substances for which the LLNA has been validated). The development of these performance standards is an initial response to this nomination, and ICCVAM is requesting comment on these performance standards.

Although we fully support the development of performance standards that expedite the validation of new protocols that are similar to previously validated methods, we reiterate our disappointment that ICCVAM/ NICEATM has chosen to apply its limited resources to the lengthy process of developing performance standards for such a narrow scope of applicability. These performance standards apply only to modifications of the "standard LLNA" that involve incorporation of non-radioactive methods of detecting lymphocyte proliferation.

A major aspect of the ICCVAM Authorization Act of 2000 (Public Law 106-545, 42 U.S.C. 285l-3) is the charge to "reduce, refine, and/or replace the use of

animals in testing where feasible." The performance standards described in this FR notice apply to modifications of the standard LLNA that do not affect the number of animals used in this method. The only conceivable reduction could occur if the availability of accepted non-radioactive methods of detection would allow more laboratories to perform the LLNA, and if they then choose the LLNA over the Guinea Pig Maximization test or the Buehler Test. The issue of how this exercise (development of performance standards with this limited applicability) addresses ICCVAM's mandate of reducing, refining or replacing the use of animals is not currently mentioned in the draft document and needs to be adequately explained.

In addition, the draft performance standards require the use of a minimum of 20 reference compounds. The criteria by which the compounds were chosen and the characteristics of the compounds are described; however, there is no justification for the requirement of such a large number of compounds for this particular method modification. The methods to which these performance standards apply will differ from the "standard LLNA" only in the method of detection of lymphocyte proliferation; therefore the element of concern is sensitivity of the detection method. All other aspects of the methods to be evaluated will be identical to the standard LLNA, including delivery and biological response. It is therefore not necessary to test representatives for every chemical class or every solvent that has been tested in the standard LLNA. The important characteristic of the reference compound is the magnitude of proliferation response that is generated, and the list of reference compounds chosen should be limited to those that represent the range of response seen with the standard LLNA.

Finally, it is the belief of the parties to this submission that the limited resources available to ICCVAM/NICEATM would be better spent on activities that would have greater impact on the reduction, refinement or replacement of animal use, such as evaluating the use of human cell lines or one of the available in vitro skin models as a replacement for the LLNA.

Sincerely,

Catherine Willett, PhD
Science Policy Advisor
Regulatory Testing Division
People for the Ethical Treatment of Animals

Sara Amundson
Executive Director
Humane Society Legislative Fund

Dr. Martin Stephens
Vice President for Animal Research Issues
Humane Society of the United States

Kristie Stoick, MPH
Research Analyst
Physicians Committee for Responsible Medicine

Sue A. Leary
President
Alternatives Research & Development Foundation

Tracie Letterman, Esq.
Executive Director
American Anti-Vivisection Society

Subject: FR Notice Comments - 73FR1360 - LLNA Peer Panel Meeting
Date: Monday, January 28, 2008 9:33 AM

Below is the result of your feedback form. It was submitted by
() on Monday, January 28, 2008 at 09:33:10

Comment_date: January 28th 2008

Prefix: Dr.

FirstName: David

LastName: Basketter

Degree: BSc DSc FRCPath

onBehalfOf: no

Title:

Department:

Company:

Country: UK

Comments: Looking at the very detailed work that has been done on reviewing potency
assessments in the LLNA, I am moved to observe that we have here a wealth of information which indicates that relative human potency can be assessed well. The scientific PRP needs to keep in mind that toxicologists working on just about all other endpoints have very much less data. Despite this, decisions on safe exposure limits are made, on a daily basis, for endpoints such as chronic tox etc, solely based on thresholds observed in rat feeding studies (or similar), where there is no validation, no correlation with human effects/potency etc., and if these were subjected to the type of rigorous review being applied to the LLNA, all of them would, without question, fail dismally. Despite limitations, the LLNA offers a good step forward in assessing skin sensitisers. Good toxicologists are those who understand the limitations of an assay, as well as its strengths.

Dr David A Basketter, BSc, DSc, CBiol, FIBiol, FRCPath

Comments on ICCVAM draft document on skin sensitisation potency

1. A very considerable body of good work has been undertaken and well documented.

2. However, human data on skin sensitisation thresholds has been given undue status as an accurate gold standard. The threshold data (no effect/lowest effect) levels are actually subject to a number of problems. These are outlined below.

3. Human threshold data for an individual allergen often (perhaps the majority of the time) represents the result of a single determination, thus there is very little information on accuracy/reproducibility.

4. As a single determination, one has no idea whether a no/low effect level is close to, or far away from, the true human threshold.

5. The protocols used to generate these human threshold data points are distinctly variable, with clear evidence of differing sensitivities between tests, most notably when comparing the human repeated insult patch test (HRIPT) with the human maximisation test. The HRIPT itself is not a standard procedure, but rather a generic name for a class of test.

6. The protocols are not always fully described, thus assumptions have to be made about certain details, notably the dosimetry (including dose per unit area and time of application, both of which are important determinants of the sensitivity of the assay).

7. The human tests use a highly outbred species, further increasing the variability of these predictive assays.

All of these points are variously made in the publications which compare directly human predictive test and LLNA skin sensitisation thresholds, but I do not see this reflected adequately in the ICCVAM document. I suppose the key point is that LLNA EC3 values, as the document indicates, do show a correlation with human thresholds, but they cannot be expected to predict the historic human data with great accuracy because that historic data is not of itself particularly precise and certainly is very far from representing a gold standard. No amount of statistical/mathematical agonising will tell us more, we just have to live with it and recognise that the human data might be good enough to indicate there is a correlation, but is not good enough to inform us about the quality of that correlation.

Please do not hesitate to ask if you have any questions.

/s/

DABMEB Consultancy Ltd, 2 Normans Road, Sharnbrook, Bedfordshire, MK44 1PR, UK
Tel/Fax: +44-1234782944; Mobile: +44-7788726937; email: david.basketter@ukonline.co.uk

Subject: MW of xylene = 107.18, or 106.12?
Date: Wednesday, May 6, 2009 8:38 PM
From: Kenneth Bogen
To: NIEHS NICEATM <niceatm@niehs.nih.gov>
(attached document: Appendix B of the Nonradioactive Murine Local Lymph Node Assay: Flow Cytometry Test Method Protocol - Draft Background Review Document - January 2008)

Dear ICCVAM Staff:

Your attached draft document lists the molecular weight of xylene as being 107.18, whereas most sources list this as 106.165 or 106.17. What is your source for the 107.18 number, and is it correct?

Best regards,

Ken

_____

Kenneth T. Bogen, DrPH DABT

Exponent
Oakland, CA 94607
www.exponent.com

The Procter & Gamble Company
Central Product Safety
Miami Valley Laboratories
P.O. Box 538707
Cincinnati, Ohio 45253-8707
www.pg.com

February 22, 2008

William S. Stokes, D.V.M., DACLAM
RADM, U.S. Public Health Service
Director, National Toxicology Program Interagency Center for the
Evaluation of Alternative Toxicological Methods
Executive Director, Interagency Coordinating Committee on
the Validation of Alternative Toxicological Methods
National Institute of Environmental Health Sciences, NIH, DHHS
P.O. Box 12233
Research Triangle Park, NC 27709

Dear Dr. Stokes

Thank you for the opportunity to review and comment on the documents prepared by ICCVAM and NICEATM related to a number of the modifications/proposed uses for the traditional LLNA that will be considered by an independent international expert panel in early March.

The teams have done a great job summarizing the available data on the LLNA and for the most part we are in agreement with the conclusions and recommendations outlined in the documents. What makes the LLNA such a valuable tool for skin sensitization hazard identification and risk assessment is that the strengths and limitations of the assay are recognized so well. I am not sure there is another toxicological test that is more understood and evaluated than the LLNA. I am certain that most experts in the field of skin allergy would agree that the older guinea pig skin sensitization test methods are considerably less understood, specifically related to their lack of evaluation through a formal validation process. Our hope is that this peer review of the LLNA will lead to a better appreciation of the LLNA and more important help researchers develop non-animal test methods for evaluating potential skin sensitizing chemicals by using the robust and quantitative natureof the LLNA as a foundation to compare new alternative methods.

For your review and consideration our LLNA experts (Cindy Ryan, Pierre Aeby, Petra Kern and myself) have prepared comments on the LLNA documents posted on the website. I hope you will find them useful and please let us know if you need any additional information.

Sincerely,

G. Frank Gerberick, Ph.D.
Research Fellow Victor Mills Society

DRAFT ICCVAM Recommendations: LLNA Potency

Comparison of LLNAEC3 values to human data:

An evaluation of the ability of the LLNA to predict the relative sensitization potency of chemicals in humans necessitates the use of human sensitization data for comparative purposes. In order for such a comparison to provide meaningful information, one must be aware of and understand the limitations in each of the datasets. The human data used in the comparison are derived from either HRIPT or HMT studies in which single test concentrations, expressed as $\mu g/cm^2$, were used for the induction phase of the test protocol. Therefore, a test concentration could be defined as the NOEL, when in reality it may just be the highest concentration tested to date which did not induce sensitization and there is a probability that higher levels would also fail to induce. This certainly could be the case if a LOEL for the particular chemical has not been identified. Indeed, it is difficult to compare LLNA EC3 concentrations against a human NOEL or an arbitrary value of the LOEL/10 (which is intended to represent an estimation of a probable induction threshold value). On one side, the LLNA data were generated using a test protocol designed to produce quantitative values with dose response information which permit the calculation of the LLNA EC3 and on the other side, the human data were generated by a variety of different human repeated insult patch test and human maximization test protocols which, by design are more qualitative in nature, and unless a series of studies were conducted, provide limited if any information on an induction dose response.

It is concerning that in the evaluation of the LLNA to predict skin sensitization potency in humans key values for the comparison are "pragmatically determined", as is indicated in lines 335-337 of the background review document "Next, the optimal EC3 value that maximized obtaining the correct skin sensitization calls for strong and weak sensitizers (using one or the other proposed decision criterion) was pragmatically determined." Similar wording is used in lines 801-804. The method or rationale for this "pragmatic determination" are not clearly evident in the document. A sound statistical approach should have been used instead and would have provided a more scientifically robust comparison.

Comparison of LLNA EC3 values to guinea pig data:

To assess the ability of the LLNA to predict skin sensitization potency in Guinea Pigs is not relevant to the purpose of this review. Guinea pig tests such as the Buehler (BT) and Guinea Pig Maximization tests (GPMT) were designed for the purpose of hazard identification and are poorly suited for potency estimations. While the ECETOC Technical Report No. 87, Contact Sensitisation: Classification According to Potency proposes methods to categorize allergenic potency based on BT and GPMT data, it demands that the study was conducted in full accord with OECD TG 406 and advises judicious interpretation of the data as does a similar European Union commission expert review. While the BT and GPMT have served the toxicology community well for many years as predictive skin sensitization hazard methods, it is important to recognize that, unlike the LLNA, neither of these tests has been formally validated by a recognized organization nor has the inter-laboratory variability been adequately investigated.

In several sections of the background review document, for examples Lines 321-324 and lines 714-717, it is indicated that for each substance with comparative LLNA and guinea pig data, potency was evaluated by comparing the LLNA EC3 concentration against the percentage of responding guinea pigs in the BT or GPMT and the associated induction concentration. Comparing LLNA EC3 concentration against the percentage of responding guinea pigs is not appropriate in our opinion and resulting data are of very different natures; the LLNA measures events associated with the induction of skin sensitization and provides objective, quantitative dose response information whereas data derived from the guinea pig tests are based on a subjective evaluation of skin responses occurring at the elicitation phase of sensitization and provides no dose response information on the induction phase.

It appears that the authors understand the difficulty of comparing LLNA EC3 values with potency classifications based on guinea pig data. In line 395 of the background review document it states that "…for substances that had more than one EC3 or guinea pig response, the geometric mean EC3 value and the weight of evidence GP classification category was used. Although the data generated by the GPMT and the BT is categorical, using the weight of evidence categorization provided some measure of a mean response across multiple studies." Considering the admitted difficulties encountered in dealing with multiple sets of guinea pig-derived data, the authors should be consistent and not make any conclusion based on such comparison.

Proposed classification categories for sensitization:

While cut-off values for potency classification are proposed based on either Buehler test and GPMT responses (Table 1-1) we would caution the use of such data in the absence of any other supporting data due to the nature of the test design. In addition, the proposed scheme uses the intradermal induction dose of the GPMT along with the % responders as the basis for classification. We believe that the topical induction concentration should be considered as it is the more relevant route of exposure and the concentration used for intradermal injection is often limited by the addition of Freund's Complete Adjuvant.

The proposed classification (as well as the one proposed by ECETOC TR No. 87) considers only data from guinea pig tests which are defined as 'positive' by the accepted TG 406 definition of a sensitizing chemical (i.e. induces 30% or 15% positive responses in the GPMT or BT respectively). It is possible that a weakly sensitizing chemical tested in a guinea pig test could elicit positive responses in 20% or 25% of the test animals in a GPMT or 10% in the BT, and would be considered as a non-sensitizer and thus would not be classified according to the proposed scheme while a chemical with any LLNA EC3 value would be assigned to one of the 2 proposed categories. Data obtained through the LLNA allows for a continuous spectrum of EC3 values and thus provides a rank ordering of relative potencies which offer more opportunities for categorization beyond two categories. And on the other side, Human and GP tests which are designed to provide yes/no answers have various threshold values creatively proposed in order to force results in the same two categories.

In the proposed two level classification scheme for sensitization potency (Table 1-1), the criteria for classification for category 1 are given as "A high frequency of occurrence…." OR "A probability of occurrence of a high sensitization rate in humans…" and for category 2 are given

as “A low or moderate frequency ….” OR “A probability of occurrence of a low to moderate sensitization rate in humans…”. The frequency of sensitization or the sensitization rate within an exposed population concerns the **prevalence** of allergic contact sensitization to a particular chemical, which is entirely different from the inherent **potency** of the chemical. Therefore the use of such criteria to classify potency is not appropriate. The likelihood of a chemical inducing skin sensitization within an exposed population (i.e. the probable sensitization rate) depends on two key elements: the intrinsic allergenic potency of the chemical AND the conditions and extent of the allergen exposure (e.g. frequency, duration, exposure conditions, etc.). Clinically, the nature, extent and duration of exposure are commonly the predominant determinants of prevalence. The relative potency of a chemical concerns the amount of chemical required to induce sensitization. In general, the more potent the allergen, the lower the dose per unit area required to induce sensitization. Prevalence data are derived from diagnostic patch testing of patients with suspect allergic contact dermatitis, often presenting with clinical disease, in dermatology clinics. The diagnostic patch test itself is designed to detect the weakest degrees of allergy by using occluded exposure conditions for 48 hours and highest allergen concentrations possible to elicit a reaction. For example, the standard patch test concentration for nickel sulfate is 2.5%. Applied in a diagnostic patch test using an 8 mm Finn chamber delivers a dose per unit area of 750 $\mu g/cm^2$, well above the identified human induction threshold of 154 $\mu g/cm^2$ (see Table 2 of Appendix A of the LLNA potency background review documents). Many times the nature of the exposure conditions leading to the induction of allergy for these patients is not clearly defined. At best the published results of thousands of such diagnostic patch tests can be used to evaluate trends in patch test reactions.

One example often used to illustrate the difference between potency and prevalence is nickel. It is a very common contact allergen with a relatively high sensitization rate in the US and Europe. However, experimental evidence indicates that nickel is a relatively weak contact allergen, with LLNA EC3 of 140 $\mu g/cm^2$ and a human induction threshold of 154 $\mu g/cm^2$ for nickel sulfate. The high prevalence is due to the wide distribution, frequent exposure and the nature of exposure, often through ‘compromised’ skin such as body piercing.

Conversely, the preservative methylchloroisothiazolinone/methylisothiazolinone (MCI/MI) is a well known contact allergen considered to be of strong to extreme potency with LLNA EC3 of 2.25 $\mu g/cm^2$ and a human induction NOEL of 1.25 $\mu g/cm^2$. In Europe, the prevalence rate of allergy to MCI/MI is stable at 1-3% of patch-tested patients. Considering the number of MCI/MI-containing cosmetics and toiletries that are on the market, the opportunities for exposure and the allergenic potency of the preservative one would expect a much higher incidence rate. The prevalence rate for this potent allergen is kept low because of regulatory guidelines/limits on the level of MCI/MI permissible in certain products, thus limiting the dose per unit area of the exposure. Thus, the clinical prevalence of the strong allergen MCI/MI is low whereas for nickel, a known weak allergen, the prevalence is considerably higher which is opposite of what would be expected if only looking at potency and not considering exposure.

The proposed two level classification scheme for sensitization potency (Table 1-1) does not accurately reflect the range of allergenic potencies that have been demonstrated by both animal and human data. LLNA EC3 values and human induction thresholds clearly span several orders of magnitude as shown by the data in Table 2 of Appendix A of the LLNA potency background

review documents. Human threshold values range from 1.25 μg/cm^2 for MCI/MI, to 250 μg/cm^2 for isoeugenol, to 2755 μg/cm^2 for farnesol, to 20,690 μg/cm^2 for benzyl benzoate. Clinical experience with allergic contact dermatitis would also indicate that discrete classes of sensitizing potency exist (Contact Derm, 2000, 42:344-348).

DRAFT ICCVAM Recommendations: LLNA Applicability Domain

Draft Recommendations – Use of the LLNA to Test Mixtures:

A dataset of 18 mixtures was evaluated, 15 of which had guinea pig data and none had human data. As a result, the LLNA data were compared to the guinea pig data. Since the database is severely limited due to the lack of human data, there is no proof that the guinea pig data would be representative of the human response. Thus, using the guinea pig data as the standard to which the LLNA data should be compared is not appropriate.

In addition, the usefulness of these data is limited further by the fact that information on the ingredients is known for only one of the 15 mixtures and 11 were tested in the LLNA in an aqueous vehicle, the performance of which is also being assessed in this same report.

High quality LLNA mixture data is published in Lalko et al. (2006), cited in section 7.6 of Addendum No. 1 to the ICCVAM report. This publication concerns the evaluation of essential oils and includes analytical data on the composition of the oils as well as LLNA data on the identified major constituents. These data should have been included in the evaluation and not just mentioned as other available scientific reports.

Since the database is severely limited due to the lack of human data, we agree with the recommendation that an assessment of the suitability of the LLNA for testing mixtures should not be conducted until a sufficient quantity of quality data become available. A similar logic of course also applies to guinea pig test methods.

Draft Recommendations – Use of the LLNA to Test Metal Compounds:

The reference dataset contains human data for 17 metal compounds representing 13 different metals. Since the allergenic potential in humans of most all of the known metals has been established, one questions the importance of or need for an assessment of the LLNA's ability to detect metal allergens. However, we agree with the recommendation that the LLNA is useful for the testing of metal compounds. Whether or not the LLNA is useful for testing nickel compounds is of limited importance as nickel is a well known human contact allergen.

In addition, since only 1 of the 14 metal compounds with LLNA and human data was tested in both in an aqueous vehicle, the comparison does not add much value to the assessment, especially in light of the fact that the performance of the LLNA using aqueous vehicles is being assessed in this same report.

Draft Recommendations – Use of the LLNA to Test Substances in Aqueous Solutions:

A dataset of 21 substances tested in aqueous solutions was evaluated, 4 of which had had human data. Since the database is severely limited due to the lack of human data, we agree with the recommendation that an assessment of the suitability of the LLNA for testing substance in

aqueous solutions should not be conducted until a sufficient quantity of quality data become available.

DRAFT ICCVAM Recommendations: LLNA Limit Dose Procedure

Draft Recommendations – Limit Dose Procedure:

We agree with the recommendation that the LLNA limit dose procedure is appropriate for hazard identification purposes.

We must point out that a 10% concentration threshold for defining non-sensitizing chemicals is not, as suggested in line 44 of the recommendation, proposed by Kimber et al. (2006) as the absolute cut-off. In the discussion section of that same paper, Kimber et al. indicate that for the purposes of that article the 10% threshold was used and that that figure "should not be regarded as inviolable." They go on to say that a case could be made for using, for instance, either 15% or 20%. In the 2005 Gerberick et al. paper (Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. Dermatitis, 16(4):157-202), compounds that did not induce a positive response at any concentration tested, with the highest concentration being at least 20% or greater, were categorized as non-sensitizing.

In addition, the 10% threshold concentration at which all which all negative results would be considered valid did not originate in the cited Kimber et al 2006 publication. The original reference is Cockshott et al., 2006, Human and Experimental Toxicology, 25:387-394 in which the performance of the LLNA was evaluated in a regulatory context. In that paper, a negative result obtained with the highest concentration tested at 10% would be considered a valid result if the positive control, a mild to moderate sensitizer, gave a positive response. In other words, a chemical which is negative at a top concentration of 10% does not represent a significant human sensitization hazard. This is similar to the definition of a non-sensitizing chemical in the Guinea Pig Maximization Test (GPMT) or Buehler test as one which induces less than 30% or 15% positive responses respectively. Therefore, if a chemical elicits positive responses in 20% or 25% of the test animals in a GPMT, it would be considered as a non-sensitizer from a regulatory perspective.

Comments on DRAFT ICCVAM Recommendations: LLNA Non-Radioactive Methods

DRAFT ICCVAM Recommendations: LLNA BrdU ELISA Procedure

We agree with the recommendation that more information and data are needed on this method in order to conduct a meaningful assessment of the BrdU ELISA procedure's performance relative to the traditional LLNA. It is especially important to have information regarding the inter-laboratory performance of this assay.

We do have one suggestion for consideration. Table 6-2 of the Background Review Documents shows a comparison of standard LLNA EC3 values and 0.5x-2x range for the performance standard chemicals and EC3 values calculated from the BrdU ELISA LLNA. Since an alternative SI cutoff for the BrdU ELISA LLNA was identified that provides greater accuracy

than an SI = 3 cutoff i.e., SI = 1.3, a comparison of BrdU ELISA EC1.3 values to standard LLNA EC3 values would be helpful.

DRAFT ICCVAM Recommendations: LLNA BrdU FC Procedure

We agree with the recommendation that more information and data are needed on this method in order to conduct a meaningful assessment of the BrdU-FC procedure's performance relative to the traditional LLNA. While the total number of chemicals tested (45) is sufficient, it is especially important to have information regarding the inter-laboratory performance of this assay. The background review document speculates that the transferability of the LLNA: BrdU-FC and the eLLNA: BrdU-FC would be similar to the traditional LLNA. However, we do not think that will be the case. Flow cytometry is not a trivial technique. It is certainly more error prone than scintillation counting and often the quality of the results is very dependant on trained personnel and precise procedures.

Only 13 of the 18 minimum performance standard reference chemicals have been tested in the LLNA BrdU-FC procedure. This may not be sufficient to assess the test performance according to the ICCVAM Performance Standards for the LLNA. In addition, rather than focusing on the number of chemicals for which the BrdU-FC procedure produced equivocal results or did not obtain 100% concordance with the ICCVAN LLNA performance standard reference chemicals, we believe that it would be of greater value to investigate potential causes for those results. Such information would provide some understanding of the limitations of the methods.

Since the purpose of this evaluation of the LLNA BrdU-FC procedure is to assess its ability to be a non-radioactive alternative to the traditional LLNA, is a comparison with Guinea Pig data justified?

The provided test protocol indicates that at least 6 mice be employed for an irritation prescreen and a possible 12 more be used for the optional quantitative irritation test. Therefore, this method has the potential to use more mice than the traditional LLNA. This requirement for greater animal usage must be taken into consideration when evaluating the BrdU-FC Procedure and it must be determined that the quality or quantity of information provided by this method exceeds that which would be obtained with the traditional LLNA. In other words, are the additional mice required by the BrdU-FC worth any possible additional information that would be gained compared to conducting a traditional LLNA?

DRAFT ICCVAM Recommendations: LLNA DA Procedure

Beyond the method to assess lymph node cell proliferation, the test protocol for the LLNA DA contains several key deviations from the OECD Test Guideline 429 recommended protocol and the Essential Test Method Components as described in the Draft ICCVAM Performance Standards for the LLNA . As indicated in the recommendation document (lines 77-79), the LLNA DA has made major modification to the traditional LLNA in both the test substance treatment and sampling schedule. Therefore, this method is outside of the requirements of the draft ICCVAM Performance Standards for the LLNA and should not be consider for validation as an LLNA alternative at this time.

Subject: New Form Results 2
Date: Tuesday, February 12, 2008 3:07 AM

Below is the result of your feedback form. It was submitted by
() on Tuesday, February 12, 2008 at 03:07:25

FirstName: Laurence

LastName: Musset

Company: OECD

Title: Principal Administrator

Phone-AreaCode:

Phone-Local3:

Phone-Last4:

Phone-Ext:

QuestionsComments: Questions from the OECD Expert Group on Sensitization

I. The approach by ICCVAM to validate the LLNA for the prediction of strong and weak skin sensitizers poses a methodological challenge. The reason is that the possibility of misclassification in humans of a substance˙s potency may negatively influence the outcome of the validation; i.e., it is possible that available HRIPT and HMT data may lead to a false human skin sensitization potency categorization. It is often difficult to correctly interpret the total dose used in the human tests due to insufficient documentation of total area dosed or possible prior patient exposure history.

In their analysis, Schneider and Akkan (2004) used the chemicals included in the 1999 ICCVAM validation as a starting point for a literature search to identify skin sensitizers for which quantitative human data on induction doses were available expressed as dose per unit area (ug/cm2). They were able to identify and assess 46 substances. They were not able to identify more substances as relevant uncertainties are related to limitations in the human data, which mostly come from older studies. First, the reporting of size of the skin area to which the test substance has been applied and of the volume of test solution used is often insufficient. In some cases, skin area and test solution volume could be deduced from information given on types of patches and application systems used. Moreover, in human HRIPT and HMT studies observed incidences for sensitization reactions depend on the concentrations applied

during both the induction and elicitation phase. Often, but not in all cases, the same concentration was applied for both phases. Otherwise, the overall outcome of the test may have been influenced by different elicitation concentrations, a factor not considered in the regression analysis.

In the evaluation performed by ICCVAM in 2008, 76 substances with quantitative human data among them 16 with negative LLNA results have been included. With respect to the points raised by Schneider and Akkan, it is important that it is described why it was possible in the current analysis to include more substances with both positive human and LLNA data (n=60) than Schneider and Akkan (n=46). Therefore, detailed information on ICCVAM˙s assessment of human dose per unit area is needed and the possibility of misclassification arising from such approach needs to be described. This is important with respect to the assessment of the rate of putative misclassification of strong/weak skin sensitizers using the human data in order to interpret the outcome of the validation study.

- Should the HMT and HRIPT data be treated as equivalent?
- Is a correction factor/uncertainty factor/safety factor of 10 the most appropriate for the extrapolation of LOAEL values to NOAEL values? Schneider & Akkan (2004) used arithmetic means for human and LLNA data except when there were discordant results with varying vehicles. The authors interpolated linearly from the LOEL to a dose corresponding to an estimated sensitization incidence of 5% (DSA05). Griem et al (2003) used LOAELs which were divided by an arbritary factor in cases of high observed incidences.
- ICCVAM analyzed 250 ug/cm2 and 500 ug/cm2 as the cut-off values for a stronger sensitizer. Has the reverse analysis been performed where the LLNA (e.g., at EC3 1% or 2%) and the GP data have been set as the standard and an optimal human cut-off calculated (does it vary between the LLNA and the GP data)?

II. Once criteria are determined for acceptability and use of human data, questions arise about the data from LLNA studies:

- Can the LLNA protocols be narrowed, e.g., by selection of solvents or choice of other test parameters to improve correlation coefficients? Is it meaningful to combine results for different solvents?
- For repeat LLNA studies for a chemical substance, which EC3 value should be selected? Should the geometric mean or the most conservative value be used?

III. How representative of sensitizers may the selection of chemicals with human data be? Does the set of chemicals analyzed by ICCVAM emphasize strong sensitizers?

IV. What are the differences between the validation approach used by Basketter, Gerberick and Kimber (BRD Appendix A) with the approach taken by ICCVAM?

V. With regard to Table 6-2, please compare and contrast the approaches taken by the various investigators represented. That is, analyze the possible sources of variability in the various approaches.

VI. Note that ICCVAM presents the variability among EC3 values for repeat LLNA tests. Can the panel estimate variability for human data points?

VII. When weighing evidence in human or animal data, what are the critical parameters to be considered?

Subject: public comment on federal register of 1/8/08 vol 73 #5 pg 1360 dhs nih
Date: Tuesday, January 8, 2008 7:05 AM

murine local lymph node assay llna test method - attn dr william stokes and sam wilson

use zero animals, not fewer animals. the testing of these materials on animals started in medieval times -l500 a.d. and we should be using more modern, more accurate methods today than torturing animals in labs. use people to test skin sensitization -- then you will get real information on the sensitization. what you are doing is torturing animals. i am sick of that torture of animals.

b. schau

February 22, 2008

Dr William S Stokes
Director, NICEATM
National Institute of Environmental Health Sciences
PO Box 12233, MD EC-17
Research Triangle Park, NC 27709

Re: **73 FR 25553; January 8, 2008; National Toxicology Program (NTP); NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments**

Dear Dr. Stokes:

These comments are submitted on behalf of People for the Ethical Treatment of Animals and the Physicians Committee for Responsible Medicine. The parties to this submission are national animal protection, health, and scientific advocacy organizations with a combined constituency of more than two million Americans who share the common goal of promoting reliable and relevant regulatory testing methods and strategies that protect human health and the environment while reducing, and ultimately eliminating, the use of animals.

Please take note of the following thoughts and transmit them to the Peer Review Panel (PRP) accordingly.

In January, 2007, (ICCVAM) received a nomination from the U.S. Consumer Product Safety Commission (CPSC) to evaluate the validation status of: (1) The murine local lymph node assay (LLNA) as a stand-alone assay for determining potency (including severity) for the purpose of hazard classification; (2) the ''cut-down'' or ''limit dose'' LLNA approach; (3) non-radiolabeled LLNA methods; (4) the use of the LLNA for testing mixtures, aqueous solutions, and metals; and (5) the current applicability domain (i.e., the types of chemicals and substances for which the LLNA has been validated).

Now more than a year later, ICCVAM is preparing for a peer review meeting to evaluate its recommendations and findings on these four items. It is unclear when final recommendations will be transmitted to federal agencies, but if ICCVAM's review of *in vitro* pyrogenicity methods is any indication, it may be at least another year.

Since this review of the LLNA and the proposed recommendations contained therein will lead to little reduction or refinement of animal use in sensitization, the resources that ICCVAM devote to this exercise should be kept to a minimum, and any forthcoming recommendations should be transmitted to agencies immediately following the Peer Review.

We have divided our comments into sections following the FR Notice:

LLNA limit dose procedures (the reduced or rLLNA) —draft Background Review Document (BRD) and other related documents

In April, 2007, ESAC issued a statement supporting the use of the rLLNA "within tiered-testing strategies to reliably distinguish between chemicals that are skin sensitizers and non-sensitizers "thereby reducing animal use by as much as 50%."[1]

In spite of the ESAC recommendations, ICCVAM has conducted its own data call in and data review. The reviewed database is comprehensive and contains a broad cross-section of the chemical universe. The performance characteristics were all above 95% (false negative and positive rates are very low or zero). Even though this additional review was largely unnecessary, we are pleased that ICCVAM's draft recommendations concluded favorably for the rLLNA procedure and urge the Peer Review Panel to concur. ICCVAM should forward recommendations regarding the use of the rLLNA to federal agencies immediately following the Peer Review.

Mixtures, metals, and aqueous solutions—draft Updated Assessment of the Validity of the LLNA for Mixtures, Metals, and Aqueous Solutions and related documents

ICCVAM has evaluated available data with respect to the use of LLNA in predicting the skin sensitization potential of mixtures, metals, and aqueous solutions. In all cases, the limited availability of data prevented a conclusive recommendation for the use of the LLNA; for metals, the LLNA is recommended only as part of a weight-of-evidence approach, which does not significantly promote a reduction in the use of animals.

Clearly this approach to expanding the applicability domain of the LLNA has not proved terribly fruitful, and we do not endorse further validation efforts in this regard, but recommend all resources are directed towards the pursuit of *in vitro* methods for this purpose.

Potency—draft BRD and related documents

Once again, ICCVAM has reviewed all availed data and come to a conclusion that is in opposition to that of other experts in the field. For more than 10 years data has been accumulating indicating the potential for the LLNA to make a determination of the sensitization potency of a chemical.[2] Several publications by Basketter and others (many of which are referenced in the BRD) as well as the eloquent argument by Basketter et al. presented in Appendix A, conclude that LLNA is appropriate for determining potency. In September 2000, the European Centre for Ecotoxicology and Toxicity of Chemicals (ECETOC) published a comprehensive review of sensitization test methods with respect to hazard identification and labeling, to determine whether the various methods are appropriate for determining relative potency and risk assessment.[3] The conclusions from this review included: (1) the LLNA is a viable and complete alternative to traditional guinea pig test

[1] http://ecvam.jrc.it/publication/ESAC26_statement_rLLNA_20070525-1.pdf

[2] Kimber I, Basketter D A. Contact sensitization: A new approach to risk assessment. Human and Ecological Risk Assessment 1997: 3: 385 - 395.

[3] ECETOC. 2000. Skin Sensitization Testing for the Purpose of Hazard Identification and Risk Assessment.

methods for the purposes of skin sensitization hazard identification, and (2) the LLNA is suitable for the determination of relative skin sensitizing potency and the adaptation of this method for derivation of comparative criteria such as EC3 values provides an effective and quantitative basis for such measurements. This report further recommends that "the LLNA is the recommended method for new assessments of relative potency and/or for the investigation of the influence of vehicle or formulation on skin sensitizing potency."

More recent work has further verified the use of the LLNA as a stand-alone method for estimating potency for regulatory purposes, including a 2005 study that concludes that there is a "clear linear relationship between LLNA-derived EC3 values and historical human skin patch data."[4] A 2007 review concludes that "The LLNA, when conducted according to published guidelines, provides a robust method for skin sensitization testing that not only provides reliable hazard identification in formation but also data necessary for effective risk assessment and risk management." In addition, a retrospective analysis of the regulatory use of the LLNA in the EU was published in 2006 and concluded that "the LLNA is satisfactory for routine regulatory use." [5]

Despite all of this, ICCVAM's review of the LLNA for potency determination does not support such a finding, even though, according to the BRD, the LLNA was better overall at predicting sensitization potency than guinea pig data. It is clear from the BRD that different data treatments result in different R^2 values, and the BRD should more clearly discuss the reasons those analysis decisions were made. Further, the BRD should explain in detail why conclusions were drawn that are opposite to that of the evidence they reference.

We urge the PRP to take into account the submission in Appendix A of the draft LLNA-potency BRD, which details why the LLNA is a scientifically appropriate method of potency determination, and the subsequent submitted comment by Dr. David Basketter, a recognized expert in the field of skin sensitization, when making its final report to ICCVAM.

Non-radioactive methods—draft BRDs and related documents

Three new methods of measuring lymphocyte proliferation have been proposed. Unlike the traditional LLNA, these new methods do not use a radioactive indicator, which could increase the use of the LLNA in facilities that cannot use radioactive material. The new methods include two variants of a bromodioxyuridine system [BrdU: ELISA and BrdU: Flow Cytometry (FC)] and the LLNA: DA.

When compared to human data, the LLNA: BrdU-FC had a higher accuracy rate, higher sensitivity, the same specificity, the same false positive rate, and a lower false negative rate than the traditional LLNA. Despite this performance, the assay does not achieve complete concordance with the proposed LLNA Performance Standards the PRP will be evaluating. This is also the case with for the LLNA-DA method, which compares identically to human data, yet

[4] Basketter et al. Predictive identification of human skin sensitization thresholds. Contact Dermatitis. 2005; 53 (5): 260-267.
[5] Cockshott et al., The local lymph node assay in practice: a current regulatory perspective. Hum Exp Toxicol 2006; 25 (7): 387-394.

falls short when compared to the traditional LLNA. While reasons for this are not clear, it is worth an examination of whether we should compare new methods to the methods they are replacing or to the endpoint of actual interest.

The BrdU: ELISA has been recommended for use by ICCVAM pending receipt of additional information and using alternative decision criteria. We support this finding. Because of the incomplete concordance between these methods and the traditional LLNA, ICCVAM qualified their acceptance and recommends a "weight-of-evidence" approach. While it is usually good scientific practice to evaluate any test method results in weight-of-evidence manner, qualifications such as these undercut the recommendations and introduce undue confusion to the reader. In our view, this gives a company a clear incentive to conduct more testing, when in reality the methods evaluated have acceptable performance and should simply be recommended.

Performance Characteristics

Although we fully support the development of performance standards that expedite the validation of new protocols that are similar to previously validated methods, we reiterate our disappointment that ICCVAM/ NICETAM has chosen to apply its limited resources to the lengthy process of developing performance standards for such a narrow scope of applicability. These performance standards apply only to modifications of the "standard LLNA" that involve incorporation of non-radioactive methods of detecting lymphocyte proliferation.

In addition, the draft performance standards require the use of a minimum of 22 reference compounds. The criteria by which the compounds were chosen and the characteristics of the compounds are described; however, there is no justification for the requirement of such a large number of compounds for this particular method modification. The methods to which these performance standards apply will differ from the "standard LLNA" only in the method of detection of lymphocyte proliferation; therefore the element of concern is sensitivity of the detection method. All other aspects of the methods to be evaluated will be identical to the standard LLNA, including delivery and biological response. It is therefore not necessary to test representatives for every chemical class or every solvent that has been tested in the standard LLNA. The important characteristic of the reference compound is the magnitude of proliferation response that is generated, and the list of reference compounds chosen should be limited to those that represent the range of response seen with the standard LLNA.

In addition, a major criterion for the selection of the above compounds is that there are Guinea pig data available; more appropriately, chemicals should be chosen on the basis of available human data.

Conclusions and Future directions

This exercise is a good example of actions undertaken by ICCVAM which result in frustration in the animal protection community. In the future we hope that ICCVAM will take a more holistic approach to determine the ways in which it spends its limited time and resources so as to ensure maximum benefit for animals in laboratories.

Several non-animal methods for estimating sensitivity are under development, including quantitative structure activity relationship (QSAR) modeling that shows a high concordance with guinea pig and LLNA data,[6] quantification of peptide reactivity, which also shows a high concordance with LLNA data,[7,8] and human cell cultures.[9,10] We urge ICCVAM to secure an interagency grant from the CPSC to fund the validation of one or more of these non-animal methods. Clearly, ICCVAM and the CPSC both benefit from the sharing of resources, as the CPSC nominated the method and ICCVAM will be tasked with the final work product.

ICCVAM should consider taking a more pro-active approach similar to the European Sens-it-iv project,[11] which involves the coordinated efforts of more than two dozen groups from industry, academia and other organizations, all working toward the common goal of developing *in vitro* methods to assess immunotoxicity.

Sincerely,

/s/

Catherine Willett, PhD
Science Policy Advisor
Regulatory Testing Division
People for the Ethical Treatment of Animals

/s/

Kristie Stoick, MPH
Scientific and Policy Advisor
Physicians Committee for Responsible Medicine

[6] Fedorowicz et al. Structure-activity models for contact sensitization. Chem Res Toxicol. 2005; 18(6): 954-969.
[7] Gerberick et al. Quantification of chemical peptide reactivity for screening contact allergens: A classification tree model approach. Toxicol. Sci. 2007; 97(2): 417-427.
[8] Natsch and Emter. Skin sensitizers induce antioxidant response element dependent genes: Application to the in vitro testing of the sensitization potential of chemicals. Tox Sci. 2008; 102(1): 110-119.
[9] Sakaguchi, et al., Development of an in vitro skin sensitization test using human cell lines; huna Cell Line Activation Test (h-CLAT) II. An inter-laboratory study of the h-CLAT. Toxicol. In vitro. 2005; 20 (5): 774-784.
[10] Schoeters et al. Microarray analyses in dendritic cells reveal potential biomarkers for chemical-induced shin sensitization. Mol. Immunol. 2007; 44(12): 3222-3233.
[11] http://www.sens-it-iv.eu/

Subject: page 53 of your five year plan
Date: Monday, May 12, 2008 6:56 PM
From: jean public
To: <niceatm@niehs.nih.gov>, (others)

membership of sac

drug industry profiteers
other industries profiteers
1 national animal protection organiztaion (who is this?)
representatives selected by nih from a college, another govt agency, intl regulatory body or other corporate profiteers

i note that the revolving door from industry pervades what is going on at this agency. and i do not believe this membership is at all a cross section of the american public. i urge that you change the membership to more clearly reflect the american public, rather than corporate profiteers.

b. sachau

Subject: 74 FR 8974; February 27, 2009
Date: Tuesday, April 14, 2009 7:31 PM
From: Kate Willett
To: NIEHS NICEATM <niceatm@niehs.nih.gov>

April 14, 2009

Dr William S Stokes
Executive Director, ICCVAM
Director, NICEATM
National Institute of Environmental Health Sciences
PO Box 12233, MD EC-17
Research Triangle Park, NC 27709

Re: **74 FR 8974; February 27, 2009; National Toxicology Program (NTP); NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); Announcement of the second meeting of the Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments**

Dear Dr. Stokes:

These comments are submitted on behalf of Physicians Committee for Responsible Medicine, People for the Ethical Treatment of Animals, the Humane Society of the United States, the Alternatives Research & Development Foundation, the American Anti-Vivisection Society, and the Doris Day Animal League. These organizations represent more than ten million Americans who share the common goal of promoting regulatory testing strategies that protect human health and the environment while reducing, and ultimately eliminating, the use of animals.

In January, 2007, ICCVAM received a nomination from the U.S. Consumer Product Safety Commission (CPSC) to evaluate the validation status of: (1) The murine local lymph node assay (LLNA) for determining potency for hazard classification; (2) the ‘‘reduced” or ‘‘limit dose’’ LLNA approach; (3) non-radiolabeled LLNA methods; (4) the use of the LLNA for testing mixtures, aqueous solutions, and metals; and (5) the applicability domain of the LLNA.

More than a year later, ICCVAM’s Peer Review Panel reviewed findings on these five items and concluded that insufficient data existed to make recommendations about non-radioactive LLNA methods or the use of the LLNA to test mixtures,

aqueous solutions and metals. The second review panel meeting scheduled for April, 2009, is intended to reevaluate these issues in light of more recent and more complete data.

The draft recommendations resulting from this second review of the LLNA have the potential to lead to reduction or refinement of animal use in sensitization in some sectors, particularly for pesticide formulations and increased use of non-radioactive detection methods. However, we are still concerned that the time and resources that ICCVAM has devoted to this exercise has detracted from serious focus on promising *in vitro* methods with potential to have a much greater impact on animal use.

Proposed applicability domain of the LLNA - mixtures, metals, and aqueous solutions

The limited availability of data or the lack of clear definition of the test substance prevented a conclusive recommendation from the previous ICCVAM review for the use of the LLNA. Draft recommendations from the current review of formulation and aqueous solutions offer a potential for expanded use, if over-classification is accepted (presumably by both the manufacturer and the regulatory Agency). In the interim, little has changed in the availability of comparative human data and we support the review's observation that there is a need to identify relevant human data and human experience in order to continue to evaluate the applicability of LLNA to mixtures and aqueous solutions. As this approach would provide the most valuable information and does not involve further animal testing, it should certainly be a priority at this time.

During this second review, ICCVAM has come to essentially the same conclusion regarding the usefulness of the LLNA for testing metals that it had in May 2008 – that the LLNA may be useful except in the case of nickel-containing compounds.

Validation status of three modified (non-radioactive) LLNA test methods

Three new methods of measuring lymphocyte proliferation have been proposed. Unlike the traditional LLNA, these new methods do not use a radioactive indicator, which could increase the use of the LLNA in facilities that cannot use radioactive material. The new protocols include two methods for detecting bromodioxyuridine incorporation [BrdU-ELISA and BrdU-Flow Cytometry (FC)] and a method for detecting ATP content (LLNA: DA).

When compared to human data, the **LLNA: BrdU-FC** had a higher accuracy rate, higher sensitivity, the same specificity, the same false positive rate, and a lower false negative rate than the traditional LLNA. In order to better understand this lack of concordance, the 2008 panel requested original records for all of the studies included in the evaluation. Despite not receiving those original records, ICCVAM proceeded with the re-evaluation of this test method and, not surprisingly, arrived at a similar conclusion; that the method may prove useful; however, recommendations for use are deferred pending release of the requested data. Not only does this represent wasted effort on the part of ICCVAM and the PRP, it continues to beg the larger question of whether it is relevant to be comparing a new method, such as the LLNA: BrdU-FC, to the traditional LLNA rather than to the endpoint of actual interest, human sensitivity.

ICCVAM has concluded that it is now appropriate to recommend the **LLNA: BrdU-ELISA** and **LLNA: DA** methods with specific limitations in the decision criteria. Substances falling within an intermediate stimulation index (SI) specified for each method would be subjected to an "integrated decision strategy in conjunction with all other available information (e.g., dose response information, statistical analyses of treated vs. control animals, peptide-binding activity, molecular weight, results from related chemicals, other testing data)." While we support this finding in general, we believe that it should be made clear that "other testing data" refers to retrospective analyses rather than initiation of additional tests in animals.

The panel also recommends that all three of these alternative detection methods be evaluated for their ability to assess mixtures, metals, and aqueous solutions concurrently with the assessment of these substances in the traditional LLNA. Since the only difference between these methods and the traditional LLNA is the method of detection, it is unlikely that there will be any differences in the applicability of these methods and the traditional LLNA with regard to mixtures, metals and aqueous solutions. Therefore, it would be highly inappropriate to perform these redundant studies, especially since there are no available data for comparison.

Conclusions and Future Directions

If, based on the Draft Recommendations from this second review, the LLNA becomes a standard for pesticides formulations and if recommendations for the non-radioactive methods allow more laboratories to perform the LLNA over the Guinea Pig Maximization test or the Buehler Test, in a best-case scenario, this will

result in a moderate reduction in animal use. ICCVAM has devoted a significant portion of its resources over the past two years to these activities and we feel this is a misappropriation of ICCVAM's limited resources and do not endorse further validation efforts in this regard. Instead, we recommend that ICCVAM's limited resources be directed toward the pursuit of *in vitro* methods for this purpose.

Several non-animal methods for estimating sensitivity are under development, including quantitative structure activity relationship (QSAR) modeling that shows a high concordance with guinea pig and LLNA data [1], quantification of peptide reactivity, which also shows a high concordance with LLNA data [2, 3], in vitro skin models [4], and human cell cultures [5, 6]. We urge ICCVAM to secure an interagency grant from the CPSC to fund the validation of one or more of these non-animal methods.

ICCVAM should consider taking a more pro-active approach similar to the European Sens-it-iv project [7], which involves the coordinated efforts of more than two dozen groups from industry, academia and other organizations, all working toward the common goal of developing in vitro methods to assess immunotoxicity.

Sincerely,

Nancy Douglas, PhD
People for the Ethical Treatment of Animals

Catherine Willett, PhD
People for the Ethical Treatment of Animals

Kristie Stoick, MPH
Physicians Committee for Responsible Medicine

Martin Stephens, PhD
The Humane Society of the United States

Sara Amundson
Humane Society Legal Fund
Doris Day Animal League

Sue Leary
Alternatives Research & Development Foundation

Tracie Letterman, Esq
American Anti-Vivisection Society

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[7] http://www.sens-it-iv.eu/

15-July-2009

Dr. William S. Stokes, Director, NICEATM, NIEHS,
P.O. Box 12233,
Mail Stop: K2–16,
Research Triangle Park, NC 27709

Re: **Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products**

Dear Dr. Stokes,

Sanofi-aventis U.S. Inc, a member of the sanofi-aventis Group, appreciates the opportunity to comment on the above-referenced report, the *Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products* and provide the following comments:

General Comments

The document is quite technical and comments will focus on sections 1-3 and section 4, testing of pesticide formulation. Sanofi-aventis acknowledges some positive approaches to the LLNA methods proposed within the report. These approaches include the reduction in the number of animals, the replacement of the guinea pig, and the avoidance of radioactive compounds, and the use of negative and positive controls for the three methodologies. While the report offers three modified methodologies for the LLNA, these methodologies do not highlight significant progress from the classical LLNA.

Specific Comments

Section 1.0 – LLNA-DA

1) In this protocol the justification for replacing the guinea pig is provided. The replacement is not mentioned for the LLNA-BrdU-FC or the LLNA-BrdU-ELISA. It could be mentioned for the other two methodologies.

2) An explanation of the use of sodium lauryl sulfate is need due to ethical reasons.

Section 2.0 – LLNA BrdU-FC

1) In this protocol the ear swelling is recommended to evaluation irritancy. The assessment would be interesting for the LLNA-DA and LLNA BrdU-ELISA or the rationale to incorporate the ear swelling in this method needs to be explained.

sanofi-aventis U.S., Corporate Regulatory Affairs Office, 4520 East West Highway, Suite 210, Bethesda, MD 20814
Tel (301) 771-4261 - www.sanofi-aventis.us

2) The difficulties of the LLNA reside in classifying compound based on decision criteria for stimulation index and in discriminating irritancy from sensitization. The LLNA BrdU-FC method might offer the ability to discriminate irritants from sensitizers but might be problematic for weak sensitizers. For this assay, no inter-laboratory studies have been performed so a great deal of work is necessary to validate this approach.

Section 3.0 – LLNA BrdU-ELISA

1) The number of animals is not homogeneous between the three methodologies (LLNA-DA: 4 mice; LLNA BrDU-FC: 4-5 mice; LLNA BrdU-ELISA: 8 mice). The inconsistency might trigger the preference to avoid LLNA-BrdU-ELISA for ethical reason.

2) The validated benchmark for positive effect in the LLNA is a stimulation index of ≥3. When a value very close to 3 is observed, standard practice is to repeat the assay to obtain either a definitive result or confirm an equivocal finding. As written, the recommendation by ICCVAM appears to discourage this practice when using the LLNA BrdU-FC. This does not appear to be related to the number of animals needed and therefore there is no obvious explanation.

Sanofi-aventis appreciates the opportunity to comment on the draft ICCVAM report and hopes the comments provided are useful in preparing the final report.

Sincerely,

/s/

Brian E. Harvey, M.D., Ph.D.
Vice President
Regulatory Policy

Appendix F3

Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) Comments

SACATM Meeting on June 18-19, 2008

The following is excerpted from the final minutes and speaker presentations of the SACATM meeting convened on June 18-19, 2008. The full meeting minutes are available online at: http://ntp.niehs.nih.gov/go/8202

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Minutes from the June 18 -19, 2008 SACATM Meeting

IX. VALIDATION STATUS OF NEW VERSIONS AND APPLICATIONS OF THE MURINE LOCAL LYMPH NODE ASSAY

A. Introduction and Overview of Proposed Methods and Applications

Minutes from the June 18 -19, 2008 SACATM Meeting

Dr. Marilyn Wind presented the *Report on the Independent Scientific Peer Review Meeting: Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA), a Test Method for Assessing the Contact Dermatitis Potential of Chemicals and Products - Introduction and Overview*, on behalf of Dr. Joanna Matheson, Co-chair of the ICCVAM Immunotoxicity Working Group. In 2007, the timeline for the ICCVAM evaluations included the nomination from the CPSC, endorsement by ICCVAM, SACATM's endorsement of the recommended high priority for ICCVAM evaluation, and preparation of six detailed draft background review documents and draft performance standards. In 2008 the LLNA peer review panel met and a report was made available. The new/updated LLNA applications and protocols reviewed by the peer review panel included: LLNA limit dose procedure; LLNA for testing mixtures, metals, and aqueous solutions; non-radioactive LLNA: DA method; non-radioactive LLNA: BrdU-FC method; non-radioactive LLNA: BrdU-ELISA method; draft ICCVAM LLNA performance standards, and use of the LLNA for potency determinations. The documents prepared by NICEATM and the ICCVAM Immunotoxicity Working Group for each new/updated LLNA application included the draft BRD, the draft ICCVAM test method recommendations, and questions for the peer review panel.

Dr. Wind gave an overview of the murine LLNA test method protocol, explaining its initial development in 1986 by Kimber *et al.* (1986), its purpose, the dose levels used, and the stimulation index (SI). The test substance is applied to mouse ears and the mice are then injected through the tail vein with radiolabeled thymidine (or an analogue of thymidine). Lymph nodes are removed and the amount of radiolabel in the lymph node is determined as a measure of lymphocyte proliferation. A test substance with a stimulation index (SI) of 3 is considered a sensitizer.

The LLNA limit dose test method protocol differs from the traditional LLNA protocol in that only a single dose, the highest dose that does not induce systemic toxicity or excessive local irritation, is used. The LLNA limit dose test method database has data from 471 studies, representing 466 unique substances. Results with the LLNA limit dose test method almost always agree with results from the traditional LLNA. The draft ICCVAM recommendation was that the LLNA limit dose procedure should be used for the hazard identification of skin sensitizing substances if dose-response information is not needed.

Dr. Wind explained that there has been a comprehensive update of available data and information regarding the current usefulness and limitations of the LLNA for assessing the skin sensitizing potential of mixtures, metals, and substances tested in aqueous solutions. Substances used for the update included 18 mixtures, 17 metal compounds represented by 13 different metals, and 21 substances tested in aqueous solutions. Evaluating the test method performance for mixtures compared to guinea pig, the LLNA has an accuracy of 53% (8/15), a sensitivity of 50% (3/6), a specificity of 56% (5/9), a false positive rate of 44% (4/9), and a false negative rate of 50% (3/6). There were no comparative data for mixtures tested in humans.

Evaluating the test method performance for substances in aqueous solutions, the LLNA had 50% accuracy, 33% sensitivity, and 100% specificity compared to human data. Comparing guinea pig data, the false positive rate was 67%. The LLNA had 50% accuracy, sensitivity, and specificity. The false positive and false negative rates were high at 50% (n = 6).

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Evaluating the test method performance for metal compounds, excluding nickel, the LLNA had 86% accuracy, 100% sensitivity, and 60% specificity compared to human data for all metal compounds (n = 14). The false positive and false negative rates were 40% and 0%, respectively. The LLNA had similar accuracy and sensitivity when compared to guinea pig data (n = 6). Based on one substance tested, the false positive rate was 100%. ICCVAM prepared draft recommendations stating that the LLNA appears useful for the testing of metal compounds, with the exception of nickel. More data are needed before a recommendation on the usefulness and limitations of the LLNA for testing mixtures and aqueous solutions will be made.

Dr. Wind reviewed the non-radiolabeled LLNA: DA test method protocol and the data from 31 substances tested by Daicel Chemical Industries. The LLNA: DA had at least 90% accuracy, sensitivity, and specificity when compared to the traditional LLNA. The draft ICCVAM-recommended use was that the LLNA: DA may be useful for identifying substances as potential skin sensitizers and non-sensitizers. The non-radiolabeled LLNA: BrdU-FC test method utilized data from 45 substances submitted by MB Research Labs. The draft ICCVAM-recommended use was that the test might be useful for identifying substances as potential skin sensitizers and non-sensitizers but more information and data are needed. The non-radiolabeled LLNA: BrdU-ELISA test method used data from 29 substances. The draft ICCVAM recommended use was that it may be useful for identifying substances as potential skin sensitizers and non-sensitizers, but more information and data are needed.

Dr. Wind reviewed the draft LLNA performance standards proposed for the assessment of versions of the LLNA that vary only from the ICCVAM-recommended LLNA by using non-radioactive vs. radioactive methods. The proposed minimum list of reference substances includes 18 substances ranging from strongly positive to strongly negative and for which there are available LLNA, guinea pig, and human data. The proposed accuracy standards are based on a chemical-by-chemical match and a set of four "optional" substances for demonstrating improved performance. She then discussed the proposed intralaboratory reproducibility standards that should be derived on four separate occasions and at least one week between tests to ensure that the tests are independent using two specified chemicals with known skin sensitizing potential.

Use of the LLNA for potency categorization as a stand-alone assay was determined using 170 substances with LLNA, human, and/or guinea pig data. The draft ICCVAM-recommended use was that the LLNA should not be considered a stand-alone test for potency categorization, but could be used in a weight-of-evidence evaluation to discriminate between strong and weak sensitizers. Dr. Wind closed her presentation with a description of the independent scientific peer panel meeting held at CPSC headquarters in March 2008 with attendance of over 50 people from five countries. The panel included experts in dermatology, toxicology, biostatistics, regulatory policy, immunology, and veterinary medicine.

B. Overview of the Panel Report

Dr. Luster presented the *Overview of the LLNA Independent Scientific Peer Review Panel Report*, starting with the charge to the panel, which was to review the draft BRDs and evaluate

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the extent to which applicable validation and acceptance criteria of toxicological test methods have been appropriately addressed. Further they were to consider the ICCVAM draft test method recommendations for proposed method uses and limitations, recommended standardized protocols, test method performance standards, and proposed future studies and was asked to comment on the extent to which they are supported by the information provided in the BRD. LLNA modifications and applications evaluated included: LLNA limit dose procedure; LLNA for testing mixtures, metals, and aqueous solutions; non-radiolabeled LLNA: DA method; non-radiolabeled LLNA: BrdU-FC method; non-radiolabeled LLNA: BrdU-ELISA method; draft ICCVAM LLNA performance standards, and the use of LLNA for potency determinations.

He reported that the panel recommended the LLNA limit dose procedure, or rLLNA, which follows the traditional LLNA protocol except for the number of doses tested, for the hazard identification of skin sensitizing chemicals when dose-response information is not required. The panel also recommended that it could be used as an initial test when dose-response information is required.

The panel agreed with the ICCVAM draft recommendation for the use of the LLNA to test mixtures, metals, and substances tested in aqueous solutions and emphasized the need for the continued accrual of information (i.e., LLNA data, comparative guinea pig and human data) for mixtures, metals, and substances tested in aqueous solutions. The panel agreed with the draft ICCVAM recommendations that the LLNA: DA, LLNA: BrdU-FC, and LLNA: BrdU-ELISA non-radiolabeled test methods may be useful for identifying substances as potential skin sensitizers and non-sensitizers, but this recommendation is contingent upon receipt of additional data and information.

Regarding performance standards, the panel agreed that the use of non-radiolabeled reagents for measuring cell proliferation is a “minor” modification of the traditional LLNA protocol. Other allowable minor modifications include sex, strain, species, animals per group, and timing of test article treatment. The panel emphasized that regardless of the modification, there is the same expectation of performance and that the test method must measure only the induction phase of the immune response. They also recommended that data be collected at the level of the individual animal, that five mice per dose group be used (until reliable power calculations are conducted), and that concurrent positive controls be run until the laboratory has extensive historical data.

Regarding accuracy standards, the current database does not support the inclusion of EC3 values as a component of the accuracy evaluation. For use in hazard identification, a modified method should be evaluated with all 22 substances on the ICCVAM list (including the four optional substances) and accuracy statistics calculated. Regarding reliability standards, the panel considered using the ECt range as appropriate for the intralaboratory reproducibility analysis. They stated that the appropriateness of the 0.5x to 2.0x EC3 range for the reference substances has not been adequately justified.

The panel agreed with ICCVAM that the LLNA should not be considered a stand-alone assay for categorization of skin sensitization potency, but rather it could be used in a weight-of-evidence evaluation to discriminate between strong and weak sensitizers. More data are needed to

determine the optimal threshold in humans for distinguishing between strong and weak sensitizers.

Dr. Fox asked about the dose of BrdU and the sacrifice time following application of the chemical for the LLNA: BrdU-FC and LLNA:BrdU-ELISA test methods. He said it is important because BrdU is cytometric and expensive. Dr. Allen said NICEATM does not have a dose per weight, only a volume, which is 200 µl per mouse, and 5 hours after BrdU administration, the lymph nodes are excised for the LLNA: BrdU-FC protocol and 24 hour post injection collected for the LLNA: BrdU-ELISA. Dr. DeGeorge said the dose is administered by the weight of the animal; it is 20 µl per gram of body weight. The concentration of the BrdU injected is 100 mg/ml. He said the kinetics that were done fall between a 2 and 10-hour range, where 5 hours is the common sacrifice time. Dr. Freeman said at his company they make a standard solution and vary the volume by the weight of the mouse. Dr. DeGeorge said the information is in the BRDs.

C. Public Comments

Dr. DeGeorge registered as a public commenter and provided an annotated handout of pages 23, 24, 33, and 34 from Dr. Wind's presentation titled, *Introduction and Overview of the Proposed Methods and Applications*. He stated that although his laboratory conducts the LLNA, he is not specifically representing his lab, but is there on the basis of his experience conducting hundreds of LLNAs with various chemicals. He stated that the IP kinetics/IV dosing of BrdU can be done, though it is technically difficult, and that BrdU is less expensive than radioactive compounds. He asked SACATM to make specific recommendations that were lacking in previous expert reviews and in the tremendous amount of work that has been presented. He noted that originally the list of performance standards included 18 substances, but it was changed to add four more substances. Two tested as false positives and two as false negatives in the original LLNA vs. modified LLNA and he questioned their inclusion as test substances. Dr. DeGeorge said today was the first he had heard that 100% results would not be necessary for the modified LLNAs to be accepted. He cited the BRDs as stating that you should conduct accuracy calculations and statistics. If 18 of 18 chemicals were correct, there would be no reason in seven separate test areas to require calculations of accuracies, selectivity, and sensitivity. That number would always be 100% and anything less would fail. He believed that the true intention is not to hold the modified LLNAs to a higher standard than the original LLNA, which had an accuracy of between 72 and 86%, depending on comparisons to guinea pig or human. With respect to the flow cytometry LLNA, originally it was designed for a wide range of chemicals and included equivocal substances. In the future, picking compounds that are not clearly positive or negative should be discouraged. He stated that now the gold standard has switched. For five of the 13 sensitizers on the performance standards reference substance list, there are data from only one LLNA study for each substance.

He further stated that there would be more data for the modified LLNA than the data to which it is being compared. He called upon SACATM to espouse criteria for validation that specify a minimum accuracy and offered 90% as a reasonable number for concordance accuracy. In the case of specificity and selectivity, he suggested 80%. He considered these values to be well above the original standards and commonly recognized as acceptable. He asked SACATM to address the test method performance standards. He cited the BRDs that discuss the use of

substitutes or alternative compounds, as long as they are robust and asked SACATM to allow them. He mentioned proposed additional studies and said it should be explicitly specified whether or not they are required because the BRD says the18 chemicals need to be tested. Regarding interlaboratory reproducibility, he said you cannot move to interlaboratory validation with animals until intralaboratory validation is completed.

Kate Willett, from PETA, congratulated ICCVAM on the speed at which the review was completed. She recognized the need for development of performance standards for the methods in general, but if the comparison is between radioactive and BrdU, then the number of reference compounds is excessive. In comparing detection methods, she suggested using only a few compounds that have highly reliable data and challenging the ends of the spectrum for testing sensitivity. She then asked ICCVAM and SACATM about plans to deal with follow-up for some of the assays. She said some assays were left with no recommendation pending additional data and it sounded like additional data would be forthcoming. She asked about ICCVAM's schedule or plan for reviewing the data, because she would like to see the review completed and have ICCVAM resources spent elsewhere.

Dr. Wind responded that more data are coming in and when they get all the data ICCVAM intends to reconvene the panel to look at the new data and make recommendations.

D. SACATM Discussion

Dr. Ehrich, a lead discussant, provided written comments that Dr. White read into the record.
"• LLNA Limit Dose Procedure: 153/153 nonsensitizing agents detected and 308/318 sensitizing agents detected. The numbers make this assay look good.
• LLNA for Testing Aqueous Solutions, Metals and Mixtures: 18 mixtures tested, some without guinea pig data for validation. 17 metals tested, 12/14 sensitizers detected with 2/5 false positives. Not enough products tested to say how good this will be for metals. 21 agents at least 20% water tested but only 4 with human data, which is not enough, so can't offer opinion about this.
• Non-radioactive LLNA protocol – the LLNA DA Test Method: performance >90% for the 19 + 10 sensitizer/nonsensitizers examined, with false positives <10%. Not sure if this would be good enough for mixtures, metals or aqueous solutions.
• Non-radioactive LLNA protocol – the LLNA BrdU-FC Test Method: Flow cytometry used, with 45 test agents. Some gave equivocal results and no multi-lab studies yet. Reference studies need work. This is promising but not ready yet.
• Non-radioactive LLNA protocol – the LLNA BrdU-ELISA Test Method: This is still in progress, 23 compounds tested with an accuracy of 83%. Not detailed protocol yet. Premature to make judgments.
• Draft ICCVAM LLNA Performance Standards: no comment.
• Use of the LLNA for Potency Determinations: Purpose unclear. Was this for a validation study?"

Dr. Brown, a lead discussant, said she was a bit overwhelmed by the amount of material and focused on the final conclusions, relying on the panel and their expertise. She was impressed with the process, the number of individuals, and the thoroughness of the report. She expressed

disappointment that more conclusive recommendations could not be made from the material and that data came in too late. She asked if there were a way to make sure the data are available before setting the meeting. Dr. Brown said she shared some of the sentiments expressed by the public, such as what are the next steps. She proposed finishing this evaluation and making concrete recommendations. Tests that do not use radioactivity should get more acceptances and it is important to get the method out and get people using it. She did not find any omissions in the document. She was unclear on the purpose of the performance standards and how they would be used. She thought it should be clear what the gold standard is when asking people to provide data. The platinum standard is really what happens in humans because that is what we are trying to mimic. She said animal data are acceptable as an alternative to human data and that it is sometimes necessary to accept small sample sizes due to the limited use of alternative test methods. Dr. Stokes responded by reiterating that ICCVAM worked very swiftly once the nomination was made. NICEATM had to create the draft BRDs because the test sponsors did not submit them. He said preparing the BRDs was a huge undertaking, and test sponsors submitting complete BRDs would minimize the total review time.

Dr. Stokes said NICEATM and ICCVAM had not anticipated the difficulty in obtaining validation data and scheduled the review expecting that the data would be readily available. He said in other countries data are not provided until there is a peer-reviewed publication. This is not the case in United States and that is why there was a delay in obtaining data. He mentioned Dr. DeGeorge's comment about his data collected over the past eight years. He explained that it was a huge undertaking in terms of time and effort to obtain the original records and they did not have sufficient time or resources. Dr. Stokes said the data have been requested, some have been received, and hopefully they will get the rest. ICCVAM plans to have another expedited peer review meeting to follow up. ICCVAM is aware of the interest in these modified LLNA protocols because of the advantages offered and they are anxious to complete the review. He said agencies use an accepted traditional method in decision-making and when there is a new proposed method they always compare the performance of the new method to the existing approved method. ICCVAM is comparing new methods to both the traditional LLNA and the traditional guinea pig test because they are what the agencies accept right now. The LLNA was accepted, not because it could predict the traditional guinea pig test so well, but because its performance for predicting human sensitizers was comparable to the traditional. They will continue to assess performance of new test methods against both the currently accepted test, as well as against existing human data and/or experience, but it depends upon the data provided. He explained that they were very fortunate in getting the most robust response from industry and mentioned that the current LLNA database includes over 400 substances. compared to 200 for the original review. He acknowledged how pleased NICEATM and ICCVAM were with the willingness of industry to contribute the data, which allowed for a much more thorough evaluation of the limit test.

Dr. Charles, a lead discussant, commended the expert panel for going through the data and coming up with recommendations in the limited timeframe. He concurred regarding the inclusion of a discussion on determining the maximum dose if only a single dose is to be used in a screen process. He said you must be able to define endpoints such as "excessive irritation." He agreed with the panel for a modifying requirement that a concurrent strong positive control not be performed for every single test. The positive control is merely telling you "yes" or "no."

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He asked about using a couple of animals, instead of five animals, and about doing the tests on a continuous basis. He asked how much additional work is needed to prove that the methodology is consistent and works. For the LLNA, he saw the need for the weak sensitizers, especially with regard to adding in a 1% SLS. He said, even with three animals there is pretty good correlation with the traditional LLNA, so we need further comment from the panel about the need for five animals. He concurred that four are probably needed, especially if there is adequate power in the alternative test systems. He suggested finding alternatives to the radioisotope methods. Regarding the number of chemicals used to validate the test method performance standards, five of them were ones he considered equivocal or only had one test performed on them. He suggested using chemicals with more robust data.

Dr. Dong, a lead discussant, said the panel did a wonderful job. The tables summarizing the power analysis for the modified LLNA methods are not as transparent as they should be. More footnotes or elaborations are needed for Tables 1-1, 3-1, 4-1 and 5-1 in the report. For example, the mean response and the standard deviation (SD) for the control group are not given in each of the tables, although they can be back calculated if one is familiar with the analysis procedure. He said the information is important because the SD of the response for the control group has a direct impact on the power calculations so long as the SD for the control group is assumed as the SD for the treatment group. But more importantly, the SD or variance of the control group seems to be vehicle-driven or vehicle-specific. For example, in the power calculation for the FC LLNA as shown in Table 4-1, the SD is much better when dimethylsulfoxide is used in the control group. Hence the power calculated was much higher, up to 95% with only five animals. If and when the SD or the variability of the response of the control group is vehicle-driven, then it is likely that the accuracy of the method could also be vehicle-driven. Dr. Dong said if it is too late to address this issue for the present analysis, then it should still be something that is worth considering for future studies.

Dr. Barile commended the peer review panel on a tremendous job with the amount of data submitted. He said the evaluation of the data apparently took more time than the deadline allowed. He found that some of the conclusions, statistical analysis, and the data presented from a scientific point of view rather confusing and in some incidences the conclusions were not consistent with the data. He said there were major changes throughout the study as chemicals were added in and out. If chemicals were taken out, that would alter the results of the analysis during the conduct of the studies, especially if the study were ongoing for many years. He found a bigger problem with the reference standards; 10 of the 22 chemicals were performed in only one study and he found them very difficult to compare. Another four had just two performance studies, making the majority of the reference standard done fewer than two times. He found confusing the standards used to describe accuracy, specificity, and sensitivity when comparing between the traditional LLNA and the nonradioactive methods. He also commented on the lack of the human data. He questioned the reporting of false positives in the BrdU-FC and was unclear as to the percentage being used. He questioned the use of optional chemicals and asked if they were false positives and false negatives to get a concordance with the traditional LLNA. He said ICCVAM should make sure that false positives and false negatives with the nonradioactive methods match the traditional LLNA. He questioned what constituted a 100 % concordance. He asked about the cost of the studies, and presumed it was high because of the number of animals and the labs that were asked to do these studies. He asked if it would have

been more feasible and cost-effective to wait for the additional information to come in, especially considering the time constraints on the peer review panel. He suggested giving the regional laboratories more time, reducing the number of studies, and getting clarification on the data that have been presented.

Dr. Stokes responded that there had been some confusion about the lack of data available to support the three modified LLNA protocols. ICCVAM did receive summary data for each substance for each test method, but did not receive individual animal data. ICCVAM typically requests quality assurance reports that can also be provided to the peer review panel. ICCVAM had summary data that allowed for calculation of sensitivity and specificity for each method, but not for examination of the variation among animals receiving the same dose of each chemical. With regard to selecting the 22 proposed reference chemicals for performance standards, the Immunotoxicity Working Group spent considerable time selecting the 18 chemicals and four additional optional chemicals. They started out looking at all of the 211 chemicals in the original validation database that were commercially available and applied the different criteria that are listed as to what characteristics the chemicals should have. They selected chemicals that did not produce equivocal responses and that had data using the traditional guinea pig methods as well as human data or experience. When they applied those criteria, it significantly reduced the number of chemicals from which to choose. The working group also wanted to provide a range of diversity in terms of the vehicles used and the chemical characteristics of each of the substances and sought to have a range of potency in terms of responses. So with only 13 positive chemicals and those kinds of criteria being applied, he explained that it was difficult to identify substances that had been evaluated in multiple LLNA studies, and as a result, some substances have only one study. He said ideally it would be better to have multiple studies for each substance. He reminded SACATM that these are draft ICCVAM recommendations and that after the meeting, ICCVAM will be taking the comments into consideration, along with public comments, and the report from the independent peer review panel. He said ICCVAM appreciated the comments, which will help them to revise and finalize the performance standards.

Dr. Barile said he was unsure what "level of accuracy" means. He suggested having numbers associated with accuracy, specificity, and sensitivity. Ninety percent accuracy would be considered acceptable; 80% sensitivity, specificity, also would be scientifically on target. He said it would make this summary and future summaries and evaluations much clearer.

Dr. Fox asked Dr. Luster to provide the biological basis of the assay from a molecular and cellular biology perspective. He said this is a cell-cycle reentry assay and asked whether or not the mitochondrial DNA is being measured at the same time. Dr. Luster responded that the assay is looking at the induction of the response, not the elicitation. The material is applied to the ear and the antigens are picked up by the dendritic cells in the dermis and translocated into the lymph node. If the particular T-cell recognizes a particular antigen, it undergoes cell proliferation. It is a T-lymphocyte proliferation event that eventually leads to the elicitation and the clinical response, hypersensitivity. He added that he does not think the mitochondrial DNA proliferate much and it is mostly nuclear DNA being measured in the assay.

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Dr. Fox stated that he wanted to know exactly what is detected biologically and then follow up with two other questions. He said in the review for the validation, the panel recommended histopathology, but it was a weak recommendation. He said this recommendation should be considered because it is consistent or parallel with the previous recommendations for five ocular irritants. He suggested establishing histopathology if ICCVAM is going to continue with the LLNA. He thought that there must be a better alternative to the LLNA, i.e., realistically there has to be a way to assess toxicity and skin irritation better than applying a chemical to the guinea pig or mouse ears and looking at them to decide on activation. He saw no mention of any alternative to using whole animals in the report and thought it would be important to discuss an *ex vivo* or non-animal alternative. He said he calculated the dose of BrdU at 2000 µg/kg, which is a huge dose that can damage the nucleus. Dr. Stokes said the dose of BrdU is 5 mg BrdU/mouse. He said a validation study is currently being planned on an *in vitro* method for sensitization that Dr. Kojima would be talking about. ICCVAM is providing input regarding the chemicals to use for the study. Dr. Kojima said it is an *in vitro* sensitization assay being developed with ECVAM and would be ready next year. Dr. Fox asked for information on the biology of the LLNA. Dr. Luster responded that they are looking at activation of dendritic cells by looking at markers of cell division; CD1 and CD86 and several others are activated. He said the panel strongly suggested that there be some histology associated with the reduced LLNA. Dr. Stokes said they could discuss this further at the next advisory meeting.

Dr. McClellan questioned the change in time period and suggested some simpler approaches to comparing BrdU to tritiated thymidine. Dr. Tice responded that in every test method evaluation ICCVAM does, they look at how reliable the method is and how accurate or relevant it is in predicting the particular event that is used for classification. With the reduced LLNA, the question was: does it perform as well as the traditional method given that you are only using one dose level rather than three? In the case of the three alternative methods, each method was compared independently against the original radioactive LLNA. Even taking into account the small changes in protocol, one of the issues to address is whether those changes were considered to be minor changes or major changes, where a major change might have an impact on the performance of the assay. In the ICCVAM guidelines on the LLNA, the OECD test guidelines, and the EPA guidelines, it specifies the use of male CBA mice. Another strain of mouse or another sex of CBA can be used if you demonstrate that it doesn't impact the performance of the assay. Performance is assessed through accuracy and reliability. Performance standards were not available at the time that the original LLNA was evaluated. Performance standards are used to help accelerate the validation of an alternative test method that is functionally and mechanistically similar to an existing test method. Had those performance standards existed, they would have been used, both in the development and evaluation of the non-radioactive methods. Considering that performance standards didn't exist then, ICCVAM is not holding those assays to those standards, but they are looking to see how they perform in that context. The working group also looked at expanding the applicability domain because the traditional LLNA is not considered useful for metals. There weren't enough data on complex mixtures and on aqueous solutions. The use of LLNA for metals was a re-evaluation compared to the radioactive methods, which might have impacted also on the nonradioactive methods. Dr. Tice explained that the panel had to work through a fairly complicated scenario. NICEATM tried to set up the test methods for the panel in sequential fashion to prepare them for what they evaluated later during the meeting.

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Dr. Wind said she wanted to make sure that everyone understood that ICCVAM knew the methods being developed were nonradioactive test methods. One of the reasons the LLNA wasn't being used more widely is that there are a number of countries where the use of radioactivity is not allowed, and, in addition, there are difficulties associated with using radioactivity. She said ICCVAM thought it was important to look at nonradioactive LLNA methods; however, they did not develop those methods. She said the methods were under development and were brought to the Immunotoxicity Working Group for review. She noted that performance standards make it easier for "me too" assays to be developed and not have to go through the same rigorous validation process as the original assay. She said the Europeans were pushing for the assay to be used as a "stand-alone." It is possible with the LLNA to make a determination of up to five different potency categories. CPSC staff felt that this was very important, particularly since under the GHS, there was an expert group examining the use of LLNA in determining classification based on potency categories. She explained that the panel addressed numerous questions, which is why is the review seems so confusing.

Dr. McClellan expressed concern that such a complex structure has been created for validating new tests. He said it will result in only a few new tests being available in 10 years and suggested occasionally stepping back from the rules.

Dr. Freeman said the discussion illuminated the issue of the roles that ICCVAM, NICEATM, the committee, and the agencies play in terms of promulgating the tests in a way that can impact our society in a regulatory fashion. Dr. McClellan agreed and said he thought this meeting had been one of the best because of the breadth of the agenda and opportunities for SACATM to provide advice.

Dr. Stokes appreciated SACATM's insights and precautionary concerns. ICCVAM has advocated, from the very beginning, communicating and interacting with assay developers. When this occurs, ICCVAM connects them with regulatory scientists who have experience in that particular toxicity endpoint to discuss validation study designs and protocols before they conduct a validation study. This interaction enables ICCVAM to work with them on the appropriate design of the study and selection of the appropriate chemicals that should be used to generate the data needed by regulatory agencies to make decisions on whether that test is acceptable for the purpose that it is proposed for. He said if you look at the number of chemicals and the number of laboratories that have been used for the data for these three methods, if the performance standards had been available for the developers to use, significantly fewer number of animals would have been used at a lot less expense. Laboratories have generated probably three times as much data as ICCVAM has proposed in the draft performance standards. He said this is ICCVAM's attempt to try to get ahead of that curve and get the performance standards out there for use by test method developers. ICCVAM routinely provides performance standards now with every new method. If performance standards had been developed in 1998, it would have benefited and expedited the development and validation of these three non-radioactive LLNA methods.

Dr. Fox concurred with Dr. McClellan in not understanding the 24-hour BrdU vs. the 5-hour BrdU. He said the half-life of BrdU is only 2 hours. He suggested ICCVAM use a different

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approach in regarding assay reviews, such as bringing the proposed assay to SACATM to get input on whether it's an appropriate assay to review or if the appropriate questions are being asked in its review. Dr. Stokes said the suggestion seemed reasonable as a way to proceed in the future, whenever possible.

Appendix F4

Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) Comments

SACATM Meeting on June 25-26, 2009

The following is excerpted from the final minutes and speaker presentations of the SACATM meeting convened on June 25-26, 2009. The full meeting minutes are available online at: http://ntp.niehs.nih.gov/go/8202

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XI. Report on the Second Meeting of the Independent Peer Review Panel: Evaluation of the Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA)

A. Presentations

Dr. Paul Brown, FDA and member of the Immuntoxicity WG, provided an introduction and overview of the proposed LLNA methods and applications. He said the traditional LLNA was reviewed by ICCVAM in 1998 and again in 2008. He outlined some of the regulatory requirements for skin sensitization evaluation that currently exist and then provided an overview of the LLNA test method protocol. The purpose of the LLNA is to identify chemical sensitizers through quantification of lymphocyte proliferation. A Stimulation Index (SI) is calculated as the ratio of radioactivity incorporated into draining auricular lymph nodes cells of treated animals to that of vehicle control animals. In 2008, the peer review panel agreed with ICCVAM that more data were needed to evaluate three modified versions of the LLNA not requiring radiolabeling and application of the LLNA for pesticide formulations, other products, and substances tested in aqueous solutions. Additional data were submitted to NICEATM and ICCVAM. The Immunotoxicity Working Group (IWG) working with NICEATM revised the draft BRDs, and ICCVAM updated the draft test method recommendations.

Dr. Paul Brown provided overviews of the protocols, some details of the test method data, and a summary of the draft ICCVAM recommendations:

- The LLNA: Daicel Adenosine Triphosphate (DA) test method with specific, defined limitations can be used to identify substances as potential skin sensitizers and nonsensitizers.
- Substances that produce SI > 1.7 and < 2.5 should be evaluated using an integrated decision strategy with all available and relevant information.
- The LLNA: Bromodeoxyuridine enzyme-linked immunosorbent assay (BrdU-ELISA) test method with specific, defined limitations can be used to identify substances as potential skin sensitizers and nonsensitizers. Substances that produced $1.3 \leq SI < 2.0$ should be evaluated using an integrated decision strategy with all available and relevant information.
- The LLNA: BrdU Flow Cytometry (FC) test method appears useful for identifying substances as potential skin sensitizers or nonsensitizers; however, more information and data are needed before ICCVAM can make a recommendation.

Regarding the applicability domain of the LLNA, Dr. Paul Brown said ICCVAM had comprehensively updated data and information on 104 pesticide formulations, 6 textile dyes, 12 natural complex substances, and 24 substances tested in aqueous solutions. Based on these data, ICCVAM had the following draft recommendations:

- The LLNA is more likely than a guinea pig test to classify a pesticide formulation as a sensitizer.
- More data are needed before a recommendation on the use of the LLNA for testing dyes can be made.
- A definitive recommendation on the use of the LLNA for testing natural, complex substances cannot be made until a larger number of known human sensitizers have been tested.

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- LLNA is more likely than a guinea pig test to classify a substance tested in an aqueous solution as a sensitizer. LLNA has utility for hazard classification of substances tested in aqueous solutions provided that the potential for possible over-classification is not a limitation.

Dr. Paul Brown said the ICCVAM Independent Scientific LLNA Peer Review Panel meeting was held April 28-29, 2009, in Bethesda, MD. The panel consisted of 15 experts from six countries.

Dr. Diggs asked about the negative aspects of over-regulation. Dr. Paul Brown said it would depend on the agency. At the Center for Drug Evaluation and Research, where drugs that will be used intentionally for benefit in humans are regulated, over-classification can have negative effects. Dr. Levine said the EPA tries not to over-label because it would dilute the utility of the labeling; people would stop paying attention to the labels. Dr. Freeman said over-classification could have a commercial impact and possibly lead to product deselection when the product has real value.

Dr. Michael Luster, West Virginia University, chaired the panel and provided highlights of the panel report. He thanked the panelists, the Evaluation Group Chairs, Drs. Michael Olson, Stephen Ullrich, and Michael Woolhiser, and the NICEATM staff. He reviewed the ICCVAM charges to the Panel and the modifications and applications to be reviewed.

Dr. Luster then presented the abbreviated highlights of the Panel's report:

- LLNA: DA - The available data and test method performance support its use to identify substances as potential skin sensitizers and nonsensitizers, with certain limitations. Based on the current validation database, multiple SI values should be used as decision criteria to identify sensitizers and nonsensitizers.
- LLNA: BrdU-ELISA - The available data and test method performance support its use to identify substances as potential skin sensitizers and nonsensitizers, with certain limitations. Based on the current validation database, multiple SI values should be used as decision criteria to identify sensitizers and nonsensitizers.
- LLNA-BrdU-FC - The database of more than 45 representative test substances yielded adequate accuracy based on results from one laboratory; intralaboratory reproducibility had also been adequately demonstrated; however, a recommendation on the validity of this test should be deferred pending an independent audit of the data and an interlaboratory validation study, both of which the Panel recommended. If both of these issues can be successfully addressed, then the assay should be considered scientifically validated as an alternative method for the traditional LLNA.
- All three of the nonradiolabeled LLNA protocols are mechanistically and functionally similar to the traditional LLNA and therefore, do not require separate test method performance standards.
- An emphasis should be made to include ear swelling measurements and/or immunophenotypic markers as an indicator of irritation for the traditional LLNA and for any modified LLNA test methods.

- Any material should be a candidate for testing in the LLNA unless there are unique physicochemical properties associated with the class of test materials that might affect its ability to interact with the normal immune processes. Therefore, the LLNA should be considered applicable to pesticide formulations, other products, and substances in aqueous solutions unless there is a biologically based rationale for exclusion.
- The Panel expressed a strong desire to avoid revalidation of the LLNA for new classes/types of test substances unless there is a biologically based rationale. If any variant of the LLNA is validated for use to test novel classes, then the findings should be relevant to the family of validated LLNA tests.

Dr. Freeman asked about using the lower cut-off values as the thresholds for positive or negative labeling, in order to make decision-making more straightforward. Dr. Luster said it was a small database, the error rate for positives was too high, and it might cause misuse of the methodology. If LLNA results are indeterminate, a guinea pig test may need to be done, but overall, fewer guinea pigs will be used and the end result will benefit animal welfare. The Panel discussed peptide reactivity as a good predictor of the LLNA, but did not make a recommendation on it. Dr. Fox expressed concern for using the adenosine triphosphate (ATP) assay, deeming it a poor assay for measuring proliferation. He questioned the BrdU methodology and suggested some alternatives. Dr. Luster said the Panel did not formally discuss ways to improve the assays.

Dr. Fox said FC is the most sensitive and promising assay and Dr. Luster agreed. Dr. Freeman asked about the cost of the LLNA using FC compared to the other assays. Dr. Luster said costs include the instrument and trained personnel. He said immunophenotyping was used separately to identify irritants from sensitizers, but was not part of the Panel's review. Dr. Freeman asked about accuracy and sensitivity of the FC compared to humans or guinea pigs. Dr. Luster said the results equivocated somewhat, but that only a few chemicals did not show the same results. Dr. Fox said a two-channel fluorescence-activated cell-sorting machine is cheaper and easier to calibrate than a scintillation counter. Dr. Luster agreed due to the cost of disposal of ^{3}H-thymidine. Dr. Marilyn Brown said it is essential to assess the LLNA in relation to human data when available, and asked about the actual use of LLNA compared to guinea pig tests. Dr. Levine said the EPA is getting a fair number of LLNAs now, which should increase when companies know it is accepted. Dr. Meyer asked about statistical expertise on the panel and about comparing continuous and percentage data. Dr. Luster said there were two statisticians and they did not discuss that issue.

Dr. Hansen asked about tracking the frequency of submissions, acceptances, and revisions by registrants. Dr. Levine said the EPA does not track submissions, but has done rejection analyses on particular studies. She will suggest tracking at EPA. Dr. Luster said the OECD might have tracking information because the original LLNAs, for which they have a large database, were developed in Europe. Dr. Freeman was unsure about the outcome of recommendations once the agencies received them, so it would be good to have such information from agencies made publicly available; it may encourage further use of the methods. Dr. Fitzpatrick asked if drug sponsors might be willing to share that information with ICCVAM. Dr. Paul Brown said the FDA does not

formally track submissions, but a number of LLNA assays have been submitted. In FDA's pre-meeting discussions, sponsors were told that the LLNA is acceptable. Assays have not been rejected unless there is a problem with the particular assay. Dr. Levine asked about mixtures that contain a small component of sensitizing material, creating the possibility of false negatives, and the potential for the interaction of components in a mixture to be a sensitizer when the individual components are not. Dr. Luster said the approach is to test the individual material, the vehicle, and the mixture separately. There are examples of interaction in mixtures that have the potential to destroy the epidermis, so it is important to test the combination. Dr. Levine asked about waiving testing on new formulations if they are fairly similar to existing formulations. Dr. Luster said it would be up to the regulatory agencies, but cautioned that formulations can change between batches and between companies. Dr. Levine suggested more limited testing on pesticide formulations, which are produced in series that vary only in active ingredients. Dr. Charles asked about the use of sodium lauryl sulfate (a potential sensitizer) pretreatment in the DA assay. Dr. Luster said the data had not been obtained, but the Panel did not think it would change the outcome of the recommendation.

B. SACATM discussion

Dr. Freeman asked whether SACATM could provide advice about the priority for the inter-laboratory validation studies for the FC assay. Dr. Stokes said ICCVAM accepts nomination for evaluation or validation of test methods and then decides a draft priority, which is presented to SACATM. In this case, SACATM is presented a proposed activity, which it discusses, decides on a priority, and makes a recommendation to ICCVAM.

Regarding false positives, Mr. Wnorowski, a lead discussant, asked if the next step would be guinea pig testing and which test would carry more weight. Dr. Levine said from regulatory point of view, the most conservative tests would be used. If the weight of evidence includes human data and there is a potential for over-prediction by the LLNA, then that would be taken into consideration in the labeling. Dr. Freeman suggested using guinea pig studies for those substances in the indeterminate range. Dr. Levine said a line is included on pesticides stating, "the product may cause allergic reaction in sensitive individuals." Companies developing consumer products may abandon them if they are deemed sensitizers, so the company must make the decision about what testing is done.

Dr. Meyer, a lead discussant, expressed concern regarding the comparison of different statistical analyses between the FC and ELISA methodologies and felt this issue should be addressed. Dr. Stokes responded that before the BRD is finalized, ICCVAM would consult with a statistician to make sure the appropriate analyses were done. Dr. Meyer asked about the behavior of different classes of compounds in different assays, especially the aqueous substances, which should not go through the stratum corneum. She asked if the sodium dodecyl sulfate pre-treatment for permeability had ever been validated. Dr. Luster said it was not included in the data from the sponsors. Dr. Meyer said such treatment might explain why the different classes of compounds performed so

differently in the tests. She asked to see the statistics on the FC test before making a recommendation.

Dr. Ehrich, a lead discussant, expressed strong support for the Panel's report. She said the LLNA DA method looked ready for release. The submitter had done the appropriate steps to meet the recommendations of the 2008 panel. She said the assay is not easy technically, which is why variability is an issue. Inter- and intra-laboratory studies have been done and she supported the Panel's conclusions. The LLNA DA is more sensitive than the ELISA test method, but the intermediate range for both test methods needs to be further defined and reevaluated. No new data were presented for the ELISA beyond the 2008 panel report. Dr. Ehrich supported giving high priority to the FC inter-laboratory studies and agreed that there should not be separate performance standards for the non-radioactive methods. Some intermediate areas still exist, but could be handled on a case-by-case basis. Additional performance standards would only add unnecessary delay to the release. She said it is important to provide non-radioactive tests, since some places do not allow radiation. Testing for mixtures, pesticide formulations, aqueous solutions, and metals is improved since the 2008 report. There are still some substances that are difficult to test, but there is no reason to continue to use radiolabeled testing in guinea pigs.

Dr. Charles, a lead discussant, generally concurred with Panel's recommendations and agreed giving a high priority to the FC testing. The use of dual ranges in the DA and ELISA assay for assessing sensitizers versus non-sensitizers could potentially place many compounds in limbo, so the decision criteria should be reassessed as more data are obtained. He concurred with the suggestion to include evaluation of ear swelling as an indicator of irritation and immunophenotypic marker assessment. The BRD formulations tested included many potential false negatives relative to the guinea pig maximization test (GPMT). He agreed that the GPMT was never fully validated for formulations and possibly under-predicts relative to the LLNA.

Dr. Barile, a lead discussant, suggested including data on accuracy, specificity, sensitivity, and performance standards that were available only in the BRDs from last year. He found it hard to make suggestions on applicability since new substances were added to the test formulations without including the performance data. He approved of the two decision criteria to allow specific cut-off points. He questioned the concern about the lack of human data, which are hard to obtain, and why comparisons with animal data are not enough. He questioned the prohibitions on using radioactivity in other countries and stated that radioactive procedures are very sensitive, though costly, and should not be discarded. He asked about the development of non-animal tests for detecting sensitizers. Dr. Stokes mentioned the human Cell Line Activation Test (h-CLAT) method undergoing validation in Japan and the peptide reactivity assays submitted for validation by Proctor and Gamble. Because of the Cosmetics Directive in Europe, which will completely ban the use of animals for repeat dose studies by 2013, there is much interest in developing non-animals methods to assess allergic contact dermatitis. Dr. Barile said he would like to see more discussion regarding the biology and mechanisms that are the bases of the tests, such as what is being tested by the LLNA, what cell types are proliferating, and which mouse strain is being used. He

suggested making the non-animal testing a priority over the FC tests. Dr. Fox suggested the compounds be tested for photoactivation and photosensitization. He agreed with Dr. Barile that non-animals methods should have the highest priority. Dr. Stokes clarified that ECVAM has the lead on three non-animal validation studies, which are a high priority in Europe. Dr. Kreysa added that ECVAM had received three submissions for non-animal test methods for skin sensitization and are planning validation studies now. Using these three test methods in a testing strategy could possibly serve as a replacement for animal tests.

Dr. Paul Brown said the FDA typically does not do non-clinical testing of drug products for photoallergencity. Topical products are usually tested in a human photoallergenicity study and a repeat patch test for allergenicity in humans. Those results determine further clinical development and assessment for hypersensitivity reactions; therefore, the FDA would eventually get definitive human data to characterize photosensitivity of a product. Dr. Fox encouraged testing for photoallergenicity and said the assay does not address it. Dr. Luster said there are LLNA data on photosensitization. Dr. Meyer encouraged the development of non-radioactive methods, which are easier to teach, and said ELISAs are easier technically to teach than FC. Dr. Corcoran asked about thresholds and the boundary between positive and non-positive responses in the LLNA and the guinea pig test. Dr. Luster said false positives were an issue with pesticide formulations. In the old GPMT, the substance was just put on the skin. Now, 1% pluronic acid can be used as detergent to increase dermal penetration of water-soluble substances. Mr. Wnorowski said the GPMT is generally considered more conservative and more likely to give false positives than the Buehler test; whereas the Buehler test tends to give a positive response less often. The sensitivity of the human test is intermediate. The LLNA is the most conservative and generates the most false positives. Many registrants consider that unacceptable and would be reluctant to label the product as a sensitizer. Dr. Corcoran hoped to hear that the LLNA identified sub-positive responses, creating a weight of evidence argument against labeling. He thought the LLNA's rate of false positives caused over-classification and could be a disincentive for its use. Mr. Wnorowski concurred. Dr. Levine said from a regulatory perspective, it is possible to eliminate the Buehler test if replaced by another test. Dr. Freeman, a member of the original LLNA review panel, did not recall that the LLNA over-predicts compared to the GPMT. He suggested for complete transparency that the final report should reflect the performance of the various tests. Dr. Stokes said ICCVAM would extract those data from the 1999 TMER. ICCVAM has done all the analyses, and the overall accuracy of ~70% was comparable to the predictivity of the LLNA for existing human data and the combined Buehler-GPMT tests for human data. The overall accuracy of the LLNA for predicting the GPMT was about 88%. The difference of 15 % could be due to over-prediction compared to the GPMT.

Mr. Wnorowski expressed concern about the limited, additional data for the pesticide formulations. Compared to the original assays on pure chemicals, these data show that the pesticide formulations appear to produce false positives in the LLNA compared to the guinea pig-based tests. Dr. Allen clarified the difference in sensitivity between the Buehler test and the GPMT. For the 22 substances for which there were comparative tests, 20 of the guinea pig tests were actually Buehler tests, so there is a question as to

whether they could have been concordant if they had been GPMTs. Strictly comparing the performance of the LLNA and the GPMT for those 22 substances, the accuracy is not great because the trend was to get a positive result more often in the LLNA. The original concern about the use of LLNA for mixtures was that the LLNA would give false negatives, but it is actually more conservative. Mr. Wnorowski agreed and expressed concern that if the LLNA is too conservative, it will not be used unless regulatory agencies require it, because of its impact on the marketing of products.

Dr. Marilyn Brown said laboratories have moved away from using the LLNA because it is the only test that uses radioactivity. Providing a LLNA test that doesn't use radioactivity would increase its use.

Dr. Freeman asked for a vote on whether NICEATM-ICCVAM should set a high priority on the inter-laboratory validation of the FC method because the only currently data are from just one laboratory. Dr. Corcoran said everything cannot be high priority and that doing the FC validation would mean that ICCVAM could not do something else. Dr. Stokes agreed and said the vote would be advice for the NTP and NICEATM to make decisions about competing priorities for limited resources. SACATM has not provided advice on nominations for validation studies for two years, and ICCVAM currently has no new nominations for validation studies. Dr. Diggs seconded the motion. SACATM voted 9 yes, 1 no (Dr. Meyer), 1 abstention (Dr. Barile), and 1 recusal (Dr. Marsman). Dr. Meyer voted against the motion because she was uncomfortable with the statistics and thought the ELISA is a better method to move forward. Dr. Barile abstained because he thought the other two tests should have equal priority and because FC is difficult to use for training and is costly. Dr. Fox suggested lowering the priority of the ATP assay because it is technically flawed. Dr. Stokes said all SACATM comments would be considered in finalizing the recommendations of the IWG and ICCVAM.

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Appendix G

Relevant Skin Sensitization Regulations and Testing Guidelines

G1 Table of Relevant Skin Sensitization Test Regulations G-3

G2 EPA Health Effects Test Guidelines OPPTS 870.2600: Skin Sensitization (March 2003) G-7

G3 ISO 10993-10: Biological Evaluation of Medical Devices Part 10: Tests for Irritation and Delayed-type Hypersensitivity (2002) G-25

G4 OECD Test Guideline 429: Skin Sensitisation – Local Lymph Node Assay (Adopted April 2002) G-27

G5 OECD Test Guideline 406: Skin Sensitisation (Adopted July 1992) G-37

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Appendix G1

Table of Relevant Skin Sensitization Test Regulations

Note to the Reader:
Regulations may be updated in the future. It is recommended that users review the most current version of all regulations identified.

Electronic versions of United States Code (U.S.C.) can be obtained at:
http://www.gpoaccess.gov/uscode/index.html

Electronic versions of the Code of Federal Regulations (CFR) can be obtained at:
http://www.gpoaccess.gov/cfr/index.html

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Skin Sensitization Testing: Relevant US Federal Laws, Regulations, Guidelines, and Recommendations				
Agency, Center, or Office	**Regulated Products**	**Statutory Requirements**	**Regulations**	**Guidelines and Recommendations**
FDA/CDER	Pharmaceuticals	Federal Food, Drug, and Cosmetic Act (U.S.C. Title 21, Chapter 9) Public Health Service Act (U.S.C. Title 42, Chapter 6A)	21 CFR 312 21 CFR 314	Guidance for Industry Immunotoxicology Evaluation of Investigational New Drugs (2002)
EPA/OPPTS	Chemicals as defined by Section 5 of the Act Pesticides	Toxic Substances Control Act (U.S.C. Title 15, Chapter 53) Federal Insecticide, Fungicide, and Rodenticide Act (U.S.C. Title 7, Chapter 6)	40 CFR 158.50 40 CFR 158.100 40 CFR 158.340 40 CFR 700-799	OPPTS 870.2600 (2003) (see **Appendix G2**)
CPSC	Consumer Products	Federal Hazardous Substances Act (U.S.C. Title 15, Chapters 1261-1278)	16 CFR 1500.3	No Specific Guidelines, Guidances, or Recommendations
OSHA	Chemicals	Occupational Safety and Health Act of 1970 (U.S.C. Title 29, Chapter 15)	29 CFR 1910.1200	No Specific Guidelines, Guidances, or Recommendations

Relevant Skin Sensitization Regulations and Guidelines Europe		
Agency, Center, or Office	**Regulated Products**	**Regulations and Directives**
EU	Dangerous Preparations (Chemicals and Chemical Mixtures)	Directive 1999/45/EC of the European Parliament and of the Council of 31 May 1999 Annex V to Directive 67/548/EEC of 27 June 1967
	Pesticides	Directive 91/414/EEC of the European Parliament and of the Council of 15 July 1991

Relevant Skin Sensitization Regulations and Guidelines International			
Organizations	**Regulated Products**	**Legal Instruments and Recommendations**	**Guidelines, Guidance, and Recmmendations**
GHS	Chemicals	GHS Part 3, Chapter 3.4	No Specific Guidelines, Guidances, or Recommendations
ISO	Medical Devices	NA	ISO 10993-10 (2002) (see **Appendix G3**)
OECD	Chemicals	NA	OECD Test Guideline 429 (2002) (see **Appendix G4**) OECD Test Guideline 406 (1992) (see **Appendix G5**)
ICH	NA	NA	No Specific Guidelines, Guidances, or Recommendations

Appendix G2

EPA Health Effects Test Guidelines OPPTS 870.2600: Skin Sensitization (March 2003)

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United States
Environmental Protection
Agency

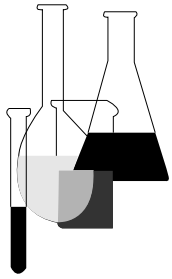
Prevention, Pesticides
and Toxic Substances
(7101)

EPA 712–C–03–197
March 2003

EPA

Health Effects Test Guidelines

OPPTS 870.2600
Skin Sensitization



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Final Guideline Release: This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on disks or paper copies: call (202) 512–0132. This guideline is also available electronically in PDF (portable document format) from EPA's Internet Web site at http://www.epa.gov/opptsfrs/home/guidelin.htm.

OPPTS 870.2600 Skin sensitization.

(a) **Scope**—(1) **Applicability**. This guideline is intended to meet testing requirements of both the Federal lnsecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. l36, *et seq*.) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background**. The source materials used in developing this harmonized OPPTS test guideline are OPPTS Harmonized Test Guidelines Series 870, Guideline 870.2600 Skin Sensitization, dated August 1998; 40 CFR 798.4100 Dermal Sensitization; OECD 406 Skin Sensitization (adopted July 1992); and OECD 429 Skin Sensitization: Local Lymph Node Assay (adopted April 2002).

(b) **Purpose**. The purpose of the selected test is to identify substances with skin sensitization potential. Determination of the potential to cause or elicit skin sensitization reactions (allergic contact dermatitis) is an important element in evaluating a substance's toxicity. Information derived from skin sensitization tests serves to identify possible hazards to a population exposed repeatedly to a test substance. Testing is not required if the test material is a known skin sensitizer. If it is suspected that the test material is a strong dermal irritant, see OPPTS 870.1000, paragraph (d)(2)(iii).

(c) **Definitions**. The following definitions apply to this test guideline. The definitions in Section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) also apply to this test guideline.

Challenge exposure is an exposure of a previously treated subject to a test substance following an induction period to elicit a contact hypersensitivity response.

Induction exposure is the administration of a test substance to the test subject with the intention of inducing contact sensitization.

Induction period is a period of at least 1 week following an induction exposure during which sensitization may develop.

Skin sensitization (allergic contact dermatitis) is an immunologically mediated cutaneous reaction to a substance. In the human, the responses may be characterized by pruritis, erythema, edema, papules, vesicles, bullae, or a combination of these. In other mammalian species, the reactions may differ and only erythema and edema may be seen.

Stimulation index (SI) is the ratio of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine (^{125}IU) incorporation into test group lymph nodes relative to that recorded for solvent/vehicle control group lymph nodes.

(d) **Test procedures**—(1) **Methods**. Any of the following test methods is considered to be acceptable:

(i) Local Lymph Node Assay (LLNA) test, or

(ii) Guinea-Pig Maximization Test (GPMT), or

(iii) Buehler test.

(2) **Choice of assays**. See OPPTS 870.1000 for a general discussion of factors to be considered prior to performing the test. In addition, the following considerations apply:

(i) The LLNA (see references in paragraphs (g)(1) through (g)(6) of this guideline) is a preferred alternative method, where applicable, to the traditional guinea pig test because it demonstrates an equivalent prediction of human allergic contact dermatitis as compared to the other sensitization tests, provides quantitative data and an assessment of dose-response, gives consideration to animal welfare concerns, and is suitable for testing colored substances. It should be recognized that there are certain testing situations that may necessitate the use of traditional guinea pig tests. The tester should note that the LLNA may not be appropriate for all types of test materials, such as certain metallic compounds, high molecular weight proteins, strong dermal irritants and materials that do not sufficiently adhere to the ear for an acceptable period of time during treatment. When using the LLNA, particular care should be taken to ensure that hydrophilic materials are incorporated into a vehicle system that wets the skin and does not immediately run off. Thus, wholly aqueous vehicles or test materials and runny liquids are to be avoided. In all instances, the tester must document that appropriate techniques were used to facilitate adherence to the mouse ear for an adequate exposure duration. It may be possible to use the LLNA to test some of these materials if appropriate techniques are used to facilitate adherence.

(ii) In situations for test materials where the LLNA is not applicable or may provide unreliable or problematic results, the GPMT or Buehler tests are recommended (see references in paragraphs (g)(7) through (g)(14) of this guideline).

(iii) Although the LLNA, GPMT, or Buehler tests are considered to be acceptable tests, it is recognized that other tests may give useful results. If other tests are used, the investigator must provide justification/reasoning for use of other procedures and methods and protocols must be provided. A positive and negative control group must be included in each test.

(e) **Test methods**—(1) **LLNA method**—(i) **Principle of the method**. The basic principle underlying the LLNA is that skin sensitizers induce proliferation of lymphocytes in the lymph nodes draining the site of chemical application. Generally, under appropriate test conditions, this proliferation is proportional to the dose applied, and provides a means of obtaining an objective, quantitative measurement of sensitization. The test measures cellular proliferation as a function of *in vivo* radioisotope incorporation

into the DNA of dividing lymphocytes. The LLNA assesses this proliferation in the draining auricular lymph nodes located in the cervical region at the bifurcation of the jugular vein. Lymphocyte proliferation in test groups is compared to that in concurrent solvent/vehicle-treated controls. A positive control is added to each assay to provide an indication of appropriate assay performance.

(ii) **Animal selection**—(A) **Sex and strain of animals**. Young adult female mice (nulliparous and non-pregnant) of the CBA/Ca or CBA/J strain should be used at age 8–12 weeks. All animals are to be age-matched (preferably within a one-week time frame). Females are used because the existing database is predominantly based on this gender. Males and other strains of mice should not be used until it is sufficiently demonstrated that significant strain-specific and/or gender-specific differences in the LLNA response do not exist.

(B) **Housing and feeding**. The temperature of the experimental animal room should be 21 ± 3 °C and the relative humidity 30–70%. When artificial lighting is used, the light cycle should be 12 hours light: 12 hours dark. For feeding, standard laboratory mouse diets are to be used with an unlimited supply of drinking water. The mice must be acclimatized for at least 5 days prior to the start of the test. Animals must be housed individually. Healthy animals are randomly assigned to control and treatment groups having statistically homogeneous body weights. The animals are uniquely identified prior to being placed on study. Although a variety of techniques exist to uniquely mark mice, any method that involves identification via ear marking (e.g., ear tags) must not be used.

(iii) **Test conditions**—(A) **Preparation of doses**. Solid test substances are to be dissolved in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Stable suspensions might also be acceptable. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance are to be prepared daily unless stability data demonstrate the acceptability of storage.

(B) **Solvent/vehicle**. The solvent/vehicle is to be selected on the basis of maximizing the test concentration while producing a solution/suspension suitable for application of the test substance. In order of preference, recommended solvents/vehicles are acetone/olive oil (4:1 v/v), *N,N*-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethyl sulfoxide, but others may be used if appropriately justified. The selected solvent/vehicle must not interfere with or bias the test result and should be selected to achieve the maximum concentration/skin exposure of the test substance. Ensure that hydrophilic materials are incorporated into a vehicle system that wets the skin and does not immediately run off. Thus, wholly aqueous vehicles are to be avoided.

(C) **Controls**. (*1*) Concurrent negative (solvent/vehicle) and positive controls are to be included in each test. In some circumstances, it may be useful to include a naive control. Except for treatment with the test substance, animals in the control groups are to be handled in an identical manner to animals of the treatment groups.

(*2*) Positive controls are used to ensure the appropriate performance of the assay. The positive control must produce a positive LLNA response at an exposure level expected to give an increase in the stimulation index (SI) of three or greater (SI ≥ 3) over the solvent or vehicle control group. The positive control dose is to be chosen such that the induction is clear but not excessive. Preferred positive control substances are hexyl cinnamic aldehyde (HCA) and mercaptobenzothiazole. There may be circumstances where, given adequate justification, other positive control substances may be used. However, benzocaine should not be used as a positive control in the LLNA.

(*3*) The positive control substance is tested in the vehicle that is known to elicit a consistent response (i.e., acetone/olive oil). If a non-standard vehicle (chemically relevant formulation) is used with a positive control, the non-standard vehicle (chemically relevant formulation) must be tested for a local lymph node response prior to the initiation of the study and the results reported.

(iv) **LLNA test procedure**—(A) **A minimum of five animals are used per dose group**. At least three consecutive doses of the test substance are to be used. A solvent/vehicle control group and a positive control group are also required. Doses are normally selected from within the concentration series 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, 0.1%. In general, dose selection is based on factors such as toxicity, solubility, irritancy and any other available information such as the results of other testing and structure-activity relationships. To avoid false negatives, test as high a concentration as possible. Generally, the maximum concentration tested is the highest achievable level that avoids overt systemic toxicity and excessive local irritation. To identify the appropriate maximum test substance dose, an initial toxicity test, conducted under identical experimental conditions except for an assessment of lymph node proliferative activity, may be necessary. To support an ability to identify a dose-response relationship, data must be collected on at least three test substance treatment doses, in addition to the concurrent solvent/vehicle control group. Where the LLNA study results are negative, the concurrent positive control must induce a SI ≥ 3 relative to its solvent/vehicle-treated control.

(B) **LLNA experimental procedure**. The LLNA experimental procedure is to be performed by appropriately trained staff as follows:

(*1*) Day 1. Record the body weight of each mouse prior to dermal applications. Apply 25 μL/ear of the appropriate dilution of the test sub-

stance, or the positive control, or the solvent/vehicle control alone to the dorsum of both ears. A positive displacement pipettor may facilitate application of the test material.

(*2*) Days 2 and 3. Repeat the application procedure as carried out on day 1.

(*3*) Days 4 and 5. No treatment.

(*4*) Day 6. Record the body weight of each mouse. Inject 250 µL of sterile phosphate buffered saline (PBS) containing 20 µCi of ^{3}H-methyl thymidine or 250 µL PBS containing 2 µCi ^{125}IU and 10^{-5} M fluorodeoxyuridine into each experimental mouse via the tail vein. Five hours later, the draining (auricular) lymph node of each ear is excised and pooled in PBS for each animal. A single cell suspension of lymph node cells (LNC) is prepared for each mouse. The single cell suspension is prepared in PBS by either gentle mechanical separation through 200-mesh stainless steel gauze or another acceptable technique for generating a single cell suspension. The LNC are washed twice with an excess of PBS and the DNA precipitated with 5% trichloroacetic acid (TCA) at 4 ^{o}C for approximately 18h.

(*5*) For the ^{3}H-methyl thymidine method, pellets are resuspended in 1 mL TCA and transferred to 10 mL of scintillation fluid. Incorporation of ^{3}H-methyl thymidine is measured by B-scintillation counting as disintegrations per minute (dpm) for each mouse and expressed as dpm/mouse. For the ^{125}IU method, the 1 mL TCA pellet is transferred directly into gamma counting tubes. Incorporation of ^{125}IU is determined by gamma counting and also expressed as dpm/mouse.

(C) **Observations**. At a minimum, observe mice once daily for any clinical signs, either of local irritation at the application site or of systemic toxicity. Weighing mice prior to treatment and at the time of necropsy will aid in assessing systemic toxicity. All observations are systematically recorded, with records being maintained for each individual mouse.

(D) **Measurements and calculation of results**. (*1*) The proliferative response of lymph node cells from the pooled lymph nodes of each individual animal is expressed as the number of radioactive disintegrations per minute (dpm) per animal, subtracting out any background dpm. Then the group mean dpm, along with an appropriate measure of inter-animal variability (i.e., mean ± standard deviation), is calculated for each test group (i.e., positive, solvent/vehicle, and any other control groups) and the solvent/vehicle group. Final results are expressed as the SI which is calculated as a ratio (i.e., SI = mean dpm of test group divided by mean dpm of solvent/vehicle control group).

(*2*) In addition to an assessment of the magnitude of the ratio estimate, SI, conduct statistical analyses which include both an overall assess-

ment (e.g. ANOVA) of the dose-response relationships and pairwise comparisons of the SIs of the test groups, positive control group and any other control group versus that of the solvent/vehicle control group. In choosing an appropriate method of statistical analysis, the investigator should be aware of possible inequality of variances and other related problems that may necessitate a data transformation or a nonparametric statistical analysis.

(v) **Data interpretation and reporting for LLNA**—(A) **Data Interpretation**. (*1*) A substance is regarded as a skin sensitizer in the LLNA if at least one concentration of the test material results in a 3-fold or greater increase in ^{3}H-methyl thymidine or ^{125}IU incorporation in the lymph node cells of test group lymph nodes relative to that recorded for solvent/vehicle control lymph nodes, as indicated by the SI. However, the magnitude of the SI should not be the sole factor used in determining the biological significance of a skin sensitization response. A quantitative assessment must be performed by statistical analysis of individual animal data in order to provide a more complete evaluation of the test substance (see paragraph (e)(1)(iv)(D)(*2*) of this guideline). Factors to be considered in evaluating the biological significance of a response or outcome of the test include the results of the SI determinations, statistical analyses, the strength of the dose-response relationship, chemical toxicity, solubility, and the consistency of the solvent/vehicle and positive control responses.

(*2*) Strong irritants may yield false positive results in the LLNA due to the initiation of a significant lymphocyte proliferation. However, the dose-response information from the assay may help to uncover a strong irritant response since, for instance, it has been shown that the proliferation induced by irritation usually results in a shallow dose-response relationship. Concurrent evaluation of ear swelling may also provide helpful information on differentiating weak sensitizers from strong irritants.

(B) **Test report**. The test report for LLNA must contain the following specific information:

(*1*) Test substance. (*i*) Identification data and CAS number, if known, and EPA registration number, if applicable;

(*ii*) Physical nature and purity;

(*iii*) Physicochemical properties relevant to the conduct of the study;

(*iv*) Stability of the test substance, if known; and

(*v*) Lot number of the test substance.

(*2*) Solvent/vehicle. (*i*) Solvent/vehicle used and its purity;

(*ii*) Justification for choice of solvent/vehicle, if appropriate; and

(*iii*) Solubility and stability of the test substance in the solvent/vehicle.

(*3*) Test animals. (*i*) Strain of mice used;

(*ii*) Acclimation information;

(*iii*) Number, age, and sex of mice;

(*iv*) Source, housing conditions, diet, etc.;

(*v*) Individual body weight of the animals at the start and end of the test, including body weight range, mean, and associated error term for each group;

(*vi*) Health and microbiological/pathogen status of the mouse; and

(*vii*) Details of animal food and water quality;

(*4*) Test conditions. (*i*) Details of test substance preparation;

(*ii*) Details of the administration of the test substance;

(*iii*) Detailed description of treatment and sampling schedules; and

(*iv*) Methods for measurement of toxicity.

(*5*) Results. (*i*) Positive and negative (solvent/vehicle) control data in tabular form;

(*ii*) Data from range-finding study, if conducted;

(*iii*) Doses used;

(*iv*) Rationale for dose level selection;

(*v*) Signs of toxicity;

(*vi*) Dpm/mouse values for each mouse within each treatment group and control group;

(*vii*) Group mean dpm/mouse and associated error term for each treatment group and control group;

(*viii*) The SI calculated, compared to the concurrent solvent/vehicle control group, for each test substance treatment dose group, the concurrent positive control group, and any other concurrent control group;

(*ix*) Individual mouse dpm data must be presented in tabular form, along with the group mean dpm, its associated error term and the SI for each dose group;

(*x*) Criteria for considering studies as positive or negative (including information on any qualitative or quantitative measure of ear swelling);

(*xi*) Dose-response relationship;

(*xii*) Statistical analyses and method applied;

(*xiii*) Concurrent and negative control data as established in the tester's laboratory; and

(*xiv*) Concurrent positive control data.

(*6*) Discussion of the results.

(*7*) Conclusions.

(*8*) The reporting requirements specified under 40 CFR Part l58 (for pesticides) and 40 CFR Part 792, Subpart J (for toxic substances) should be followed.

(2) **GPMT and Buehler Methods**—(i) **Principle of the test methods**. Following initial exposure to a test substance, the animals are subjected, after a period of not less than 1 week, to a challenge exposure with the test substance to establish whether a hypersensitive state has been induced. Sensitization is determined by examining the reaction to the challenge exposure and comparing this reaction with that of the initial induction exposure. The test animals are initially exposed to the test substance by intradermal and/or epidermal application (induction exposure). Following a rest period of 10 to 14 days (the induction period), during which an immune response may develop, the animals are exposed to a challenge dose. The extent and degree of skin reaction to the challenge exposure is compared with that demonstrated by control animals that undergo sham treatment during induction and then receive the challenge exposure.

(ii) **Animal selection**—(A) **Species and strain**. The young adult guinea pig is preferred. Young adult commonly used laboratory strains must be employed.

(B) **Housing and feeding**. The temperature of the experimental animal room should be 20 ± 3 °C with the relative humidity 30–70 percent. Where the lighting is artificial, the sequence should be 12 h light/12 h dark. Conventional laboratory diets may be used with an unlimited supply of drinking water. It is essential that guinea pigs receive an adequate amount of ascorbic acid.

(C) **Number and sex**. The number and sex will depend on the method chosen. Either sex may be used in the Buehler test and the GPMT. If females are used, they must be nulliparous and not pregnant. The Buehler test recommends using a minimum of 20 animals in the treatment and at least 10 as controls. At least 10 animals in the treatment group and 5 in the control group must be used with the GPMT, with the stipulation that if it is not possible to conclude that the test substance is a sensitizer after using fewer than 20 test and 10 control guinea pigs, the testing of

additional animals to give a total of at least 20 test and 10 control animals is strongly recommended

(D) **Control animals**. (*2*) Every 6 months, assess the sensitivity and reliability of the experimental technique in naive animals by the use of positive control substances known to have mild-to-moderate skin-sensitizing properties. In a properly conducted test, a response of at least 30 percent in an adjuvant test and at least 15 percent in a nonadjuvant test is expected for mild-to-moderate sensitizers. Preferred substances are hexylcinnamic aldehyde (CAS No.101–86–0), mercaptobenzothiazole (CAS No. 149–30–4), benzocaine (CAS No. 94–09–7), dinitro-chloro-benzene (CAS No. 97–00–7), or DER 331 epoxy resin (CAS No. 25068–38–6). There may be circumstances where, given adequate justification, other control substances meeting the above criteria may be used.

(*2*) To ensure that the response to the challenge reaction in treated animals is truly of allergic origin and not due to skin irritancy, a sham-treated vehicle-only control is included in the test strategy. This sham-treated control group is treated in exactly the same manner as the test animals, except that during the induction phase the test article is omitted. The selected vehicle must not interfere or alter the test results.

(E) **Dose levels**. The dose level will depend on the test method selected. In the Buehler test, select the concentration of the induction dose such that it is high enough to cause mild irritation, and the challenge dose such that it is the highest non-irritating concentration. In the GPMT, the concentration of the induction dose must be well tolerated systemically, and must be high enough to cause mild-to-moderate skin irritation; the GPMT challenge dose must use the highest non-irritating concentration.

(F) **Observation of animals**. (*1*) Skin reactions are to be graded and recorded after the challenge exposures at the time specified by the methodology selected. This is usually at 24 and 48 hours. Additional notations are to be made as necessary to fully describe unusual responses.

(*2*) Regardless of the test method selected, initial and terminal body weights must be taken and recorded.

(G) **Procedures**. The procedures to be used are those described by the test method chosen. Brief summaries are given here, but the tester is referred to the original literature for more complete guidance on conducting the Buehler test (see references in paragraphs (g)(7) through (g)(10) of this guideline) or the GPMT (see references in paragraphs (g)(11) through (g)(14) of this guideline).

(*1*) The Buehler test uses topical administration via a closed patch on days 0, 6–8, and 13–15 for induction, with topical challenge of the untreated flank for 6 hours on day 27–28. Readings are made approximately 24 hours alter removing the challenge patch, and again 24 hours

after that. If the results are equivocal, the animals may be rechallenged one week later, using either the original control group or a new control group for comparison.

(*2*) The GPMT uses intradermal injection with and without Freund's complete adjuvant (FCA) for induction, followed on days 5–8 by topical irritation/induction, followed by topical challenge for 24 hours on day 20–22. Readings are made approximately 24 hours after removal of the challenge dose, and again after another 24 hours. As with the Buehler test, if the results are equivocal, the animals may be rechallenged 1 week later. If only 10 animals were used initially and gave equivocal results, the use of an additional 10 experimental and 5 control animals is strongly recommended.

(*3*) Blind reading of both test and control animals is recommended.

(*4*) Removal of the test material is accomplished with water or an appropriate solvent, without altering the existing response or the integrity of the epidermis.

(*5*) Hair is removed from the site of application by clipping, shaving, or possibly by depilation, depending on the test selected.

(iii) **Data and reporting for GPMT and Buehler Methods**. Data must be summarized in tabular form, showing for each individual animal the skin reaction, results of the induction exposure, and the challenge exposure at times indicated by the method chosen. As a minimum, the erythema and edema must be graded and any unusual finding must be recorded.

(A) **Evaluation of the results**. The evaluation of results will provide information on the proportion of each group that became sensitized and the extent (slight, moderate, severe) of the sensitization reaction in each individual animal.

(B) The following specific information is to be reported for the GPMT and Buehler Methods.

(*1*) A description of the method used and the commonly accepted name.

(*2*) Information on the positive control study, including the positive control substance used, the method used, and the time conducted.

(*3*) The number, species, strain, age, source, and sex of the test animals.

(*4*) Individual body weights of the animals at the start of the test and at the conclusion of the test.

(*5*) A brief description of the grading system.

(*6*) Each reading made on each individual animal.

(*7*) The chemical identification and relevant physicochemical properties of the test substance.

(*8*) Manufacturer, source, purity, and lot number of test substance.

(*9*) Physical nature, and, where appropriate, concentration and pH value for the test substance.

(*10*) The vehicles used for induction and challenge and justification for their use, if other than water or physiological saline. Any material that might reasonably be expected to react with or enhance or retard absorption of the test substance must be reported.

(*11*) The total amount of test substance applied for induction and challenge, and the technique of application in each case.

(*12*) Description of any pre-test conditioning, including diet, quarantine and treatment of disease.

(*13*) Description of caging conditions including number (and any change in number) of animals per cage, bedding material, ambient temperature and humidity, photoperiod, and identification of diet of test animals.

(*14*) Histopathological findings, if any.

(*15*) Discussion of results.

(*16*) A list of references cited in the body of the report, i.e., references to any published literature used in developing the test protocol, performing the testing, making and interpreting observations, and compiling and evaluating the results.

(*17*) The reporting requirements as specified under 40 CFR Part l58 (for pesticides) and 40 CFR Part 792, Subpart J (for toxic substances) should be followed

(f) **Screening tests**. The mouse ear swelling test (MEST) (see references in paragraphs (g)(15) through (g)(18) of this guideline) may be used as a screening test to detect moderate to strong sensitizers. If a positive result is seen in this assay, the test substance may be designated a potential sensitizer, and it may not be necessary to conduct a further test in guinea pigs. If the MEST does not indicate sensitization, the test substance should not be designated a nonsensitizer without confirmation in an accepted test using guinea pigs or LLNA if appropriate.

(g) **References**. The following references should be consulted for additional background information on this test guideline.

(1) *The Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals/Compounds.* Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), National Institutes of Environmental Health Sciences, NIH Publication No. 99–4494.3 (1999). (Document available at http://iccvam.niehs.nih.gov/methods/llnadocs/llnarep.pdf.) Description and picture of auricular lymph node dissection available at at http://iccvam.niehs.nih.gov/methods/llnadocs/LLNAProt.pdf.

(2) Kimber, I. et al. The murine local lymph node assay for identification of contact allergens: a preliminary evaluation of in situ measurement of lymphocyte proliferation. *Contact Dermatitis* 21:215–220 (1989)

(3) Kimber, I. et al. Identification of contact allergens using the murine local lymph node assay: comparisons with the Buehler Occluded Patch Test in guinea pigs. *Journal of Applied Toxicology* 10:173–180 (1990).

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(5) Basketter, D.A. et al. Interlaboratory evaluation of the local lymph node assay with 25 chemicals and comparison with guinea pig test data. *Toxicology Methods* 1:30–43 (1991).

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(7) Buehler, E.V. Delayed contact hypersensitivity in the guinea pig, *Archives of Dermatology* 91:171–177 (1965).

(8) Ritz, H.L. and Buehler, E.V. Planning, conduct and interpretation of guinea pig sensitization patch test in *Current Concepts in Cutaneous Toxicity*, ed. V. Drill and P. Lazar. Academic, New York, NY. pp. 25–42 (l980).

(9) Buehler, L.V. Occ1usive patch method for skin sensitization in guinea pigs: the Buehler method. *Food and Chemical Toxicology* 32:97–101 (1994).

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(11) Magnusson, B. and Kligman, A.M. The identification of contact allergens by animal assay. The guinea pig maximization test. *Journal of Investigative Dermatology* 52: 268–276 (1969).

(12) Magnusson, B. and Kligman, A.M. *Allergic contact dermatitis in the guinea pig.* Charles C. Thomas, Springfield, IL (1970).

(13) Magnusson, B. Identification of contact sensitizers by animal assay. *Contact Dermatology* 6:46 (1980).

(14) Magnusson, B. et al. Determination of skin sensitization potential of chemicals. Predictive testing in guinea pigs. *Arbete och Halsa:* 26(E) (1979).

(15) Gad, S.C. et al. Development and validation of an alternative dermal sensitization test: the mouse ear swelling test (MEST). *Toxicology and Applied Pharmacology 8* 4:93–114 (1986).

(16) Maisey, J. and Miller, K., Assessment of the ability of mice fed on Vitamin-A supplemented diet to respond to a variety of potential contact sensitizers. Contact Dermatitis 15:17–23 (l986).

(17) Thorne, P.S. et al., The noninvasive mouse ear swelling assay, I. Refinements for detecting weak contact sensitizers. *Fundamental and Applied Toxicology* 7:790–806 (1991).

(18) Thorne, P.S. et al. The noninvasive mouse ear swelling assay. II. Testing the contact sensitizing potency of fragrances. *Fundamental and Applied Toxicology*7:807–820 (1991).

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Appendix G3

International Organization for Standardization - ISO 10993-10: Biological Evaluation of Medical Devices Part 10: Tests for Irritation and Delayed-type Hypersensitivity (2002)

Document available from the ISO website:

http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=33364

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Appendix G4

OECD Test Guideline 429: Skin Sensitisation – Local Lymph Node Assay (Adopted April 2002)

Note: An updated version of this test guideline was approved by OECD's Working Group of National Coordinators for Test Guideline Programme in March 2010 and is expected to be formally updated by September 2010

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OECD/OCDE **429**

Adopted:
24th April 2002

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Skin Sensitisation: Local Lymph Node Assay

INTRODUCTION

1. The OECD Test Guideline Programme periodically reviews progress in test method development and refinement, both in terms of scientific advances and animal welfare, to determine whether existing Test Guidelines should be updated and whether new Guidelines should be developed. Toward that end, a new assay for the determination of skin sensitisation in the mouse, the Local Lymph Node Assay (LLNA) has been sufficiently validated and accepted to justify its adoption as a new Test Guideline (1)(2)(3). This is the second Guideline to be promulgated for assessing skin sensitisation potential of chemicals in animals. The other Guideline (406) utilises guinea pig tests, notably the guinea pig maximisation test and the Buehler test (4).

2. The LLNA provides certain advantages with regard to both scientific progress and animal welfare. It studies the induction phase of skin sensitisation and provides quantitative data suitable for dose response assessment. The details of the validation of the LLNA and a review of the associated work have been published (5)(6)(7)(8). In addition, it should be noted that the mild/moderate sensitisers, which are recommended as suitable positive control substances for guinea pig test methods, are also appropriate for use with the LLNA (6)(8)(9).

INITIAL CONSIDERATIONS

3. The LLNA provides an alternative method for identifying skin sensitising chemicals and for confirming that chemicals lack a significant potential to cause skin sensitisation. This does not necessarily imply that in all instances the LLNA should be used in place of guinea pig tests, but rather that the assay is of equal merit and may be employed as an alternative in which positive and negative results generally no longer require further confirmation.

4. The LLNA is an *in vivo* method and, as a consequence, will not eliminate the use of animals in the assessment of contact sensitising activity. It has, however, the potential to reduce the number of animals required for this purpose. Moreover, the LLNA offers a substantial refinement of the way in which animals are used for contact sensitisation testing. The LLNA is based upon consideration of immunological events stimulated by chemicals during the induction phase of sensitisation. Unlike guinea pig tests the LLNA does not require that challenged-induced dermal hypersensitivity reactions be elicited. Furthermore, the LLNA does not require the use of an adjuvant, as is the case for the guinea pig maximisation test. Thus, the LLNA reduces animal distress. Despite the advantages of the LLNA over traditional guinea pig tests, it should be recognised that there are certain limitations that may necessitate the use of traditional guinea pigs tests (e.g., false negative findings in the LLNA with certain metals, false positive findings with certain skin irritants)(10).

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PRINCIPLE OF THE TEST

5. The basic principle underlying the LLNA is that sensitisers induce a primary proliferation of lymphocytes in the lymph node draining the site of chemical application. This proliferation is proportional to the dose applied (and to the potency of the allergen) and provides a simple means of obtaining an objective, quantitative measurement of sensitisation. The LLNA assesses this proliferation as a dose-response in which the proliferation in test groups is compared to that in vehicle treated controls. The ratio of the proliferation in treated groups to that in vehicular controls, termed the Stimulation Index, is determined, and must be at least three before a test substance can be further evaluated as a potential skin sensitiser. The methods described here are based on the use of radioactive labelling to measure cell proliferation. However, other endpoints for assessment of proliferation may be employed provided there is justification and appropriate scientific support, including full citations and description of the methodology.

DESCRIPTION OF THE ASSAY

Selection of animal species

6. The mouse is the species of choice for this test. Young adult female mice of CBA/Ca or CBA/J strain, which are nulliparous and non-pregnant, are used. At the start of the study, animals should be between 8-12 weeks old, and the weight variation of the animals should be minimal and not exceed 20% of the mean weight. Other strains and males may be used when sufficient data are generated to demonstrate that significant strain and/or gender-specific differences in the LLNA response do not exist.

HOUSING AND FEEDING CONDITIONS

7. Animals should be individually housed. The temperature of the experimental animal room should be 22ºC (+ 3ºC). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

PREPARATION OF ANIMALS

8. The animals are randomly selected, marked to permit individual identification (but not by any form of ear marking), and kept in their cages for at least 5 days prior to the start of dosing to allow for acclimatisation to the laboratory conditions. Prior to the start of treatment all animals are examined to ensure that they have no observable skin lesions.

Reliability check

9. Positive controls are used to demonstrate appropriate performance of the assay and competency of the laboratory to successfully conduct the assay. The positive control should produce a positive LLNA response at an exposure level expected to give an increase in the stimulation index (SI) >3 over the negative control group. The positive control dose should be chosen such that the induction is clear but not excessive. Preferred substances are hexyl cinnamic aldehyde (CAS No 101-86-0) and mercaptobenzothiazole (CAS No 149-30-4). There may be circumstances in which, given adequate justification, other control substances, meeting the above criteria, may be used. While ordinarily a positive control group may be required in each assay, there may be situations in which test laboratories will have available historic positive control data to show consistency of a satisfactory response over a six-month or

more extended period. In those situations, less frequent testing with positive controls may be appropriate at intervals of no greater than 6 months. Although the positive control substance should be tested in the vehicle that is known to elicit a consistent response (e.g., acetone:olive oil), there may be certain regulatory situations in which testing in a non-standard vehicle (clinically/chemically relevant formulation) will also be necessary. In such situations the possible interaction of a positive control with this unconventional vehicle should be tested.

TEST PROCEDURE

Number of animals and dose levels

10. A minimum of four animals is used per dose group, with a minimum of three concentrations of the test substance, plus a negative control group treated only with the vehicle for the test substance, and a positive control, as appropriate. In those cases in which individual animal data are to be collected, a minimum of five animals per dose group are used. Dose and vehicle selection should be based on the recommendations given in reference (2). Doses are selected from the concentration series 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5% etc. Existing acute toxicity and dermal irritation data should be considered, where available, in selecting the three consecutive concentrations so that the highest concentration maximises exposure whilst avoiding systemic toxicity and excessive local skin irritation (2)(11). Except for absence of treatment with the test substance, animals in the control groups should be handled and treated in a manner identical to that of animals in the treatment groups.

11. The vehicle should be selected on the basis of maximising the test concentrations and solubility whilst producing a solution/suspension suitable for application of the test substance. In order of preference, recommended vehicles are acetone/olive oil (4:1 v/v), dimethylformamide, methyl ethyl ketone, propylene glycol and dimethyl sulphoxide (2)(10), but others may be used if sufficient scientific rationale is provided. In certain situations it may be necessary to use a clinically relevant solvent or the commercial formulation in which the test substance is marketed as an additional control. Particular care should be taken to ensure that hydrophilic materials are incorporated into a vehicle system, which wets the skin and does not immediately run off. Thus, wholly aqueous vehicles are to be avoided.

Experimental schedule

12. The experimental schedule of the assay is as follows:

- *Day 1:*
  Individually identify and record the weight of each animal. Open application of 25µL of the appropriate dilution of the test substance, the vehicle alone, or the positive control (as appropriate), to the dorsum of each ear.
- *Days 2 and 3:*
  Repeat the application procedure carried out on day 1.
- *Days 4 and 5* :
  No treatment.
- *Day 6* :
  Record the weight of each animal. Inject 250µL of phosphate-buffered saline (PBS) containing 20 µCi (7.4e+5 Bq) of ^{3}H-methyl thymidine into all test and control mice via the tail vein. Alternatively inject 250 µL PBS containing 2 µCi (7.4e + 4 Bq) of ^{125}I-iododeoxyuridine and 10^{-5}M fluorodeoxyuridine into all mice via the tail vein. Five hours (5 h) later, the animals are killed. The draining auricular lymph nodes from each ear are excised and pooled in PBS for each experimental group (pooled treatment group approach);

alternatively pairs of lymph nodes from individual animals may be excised and pooled in PBS for each animal (individual animal approach). Details and diagrams of the node identification and dissection can be found in Annex I of the ICCVAM Immunotoxicology Working Group LLNA Protocol (10).

Preparation of cell suspensions

13. A single cell suspension of lymph node cells (LNC) either from pooled treatment groups or bilaterally from individual animals is prepared by gentle mechanical disaggregation through 200 µm-mesh stainless steel gauze. Lymph node cells are washed twice with an excess of PBS and precipitated with 5% trichloroacetic acid (TCA) at 4°C for 18h(2). Pellets are either re-suspended in 1 mL TCA and transferred to scintillation vials containing 1.0 mL of scintillation fluid for ^{3}H-counting, or transferred directly to gamma counting tubes for ^{125}I-counting.

Determination of cellular proliferation (incorporated radioactivity)

14. Incorporation of ^{3}H-methyl thymidine is measured by β-scintillation counting as disintegrations per minute (DPM). Incorporation of ^{125}I-iododeoxyuridine is measured by ^{125}I-counting and also is expressed as DPM. Depending on the approach used, the incorporation will be expressed as DPM/treatment group (pooled approach) or DPM/animal (individual approach).

OBSERVATIONS

Clinical observations

15. Animals should be carefully observed once daily for any clinical signs, either of local irritation at the application site or of systemic toxicity. All observations are systematically recorded with individual records being maintained for each animal.

Body weights

16. As stated in paragraph 12, individual animal body weights should be measured at the start of the test and at the scheduled kill of the animals.

CALCULATION OF RESULTS

17. Results are expressed as the Stimulation Index (SI). When using the pooled approach, the SI is obtained by dividing the pooled radioactive incorporation for each treatment group by the incorporation of the pooled vehicle control group; this yields a mean SI. When using the individual approach, the SI is derived by dividing the mean DPM /mouse within each test substance group and the positive control group by the mean DPM/mouse for the solvent/vehicle control group. The average SI for vehicle treated controls is then 1.

18. Use of the individual approach to calculate the SI will enable the performance of a statistical analysis of the data. In choosing an appropriate method of statistical analysis, the investigator should maintain an awareness of possible inequalities of variances and other related problems that may necessitate a data transformation or a non-parametric statistical analysis. An adequate approach for interpreting the

data is to evaluate all individual data of treated and vehicle controls, and derive from these the best fitting dose response curve, taking confidence limits into account (10)(12)(13). However, the investigator should be alert to possible "outlier" responses for individual animals within a group that may necessitate the use of an alternative measure of response (e.g. median rather than mean) or elimination of the outlier.

19. The decision process with regard to a positive response includes a stimulation index ≥ 3, together with consideration of dose-response and, where appropriate, statistical significance (3)(6)(10)(13)(14).

20. If it is necessary to clarify the results obtained, consideration should be given to various properties of the test substance, including whether it has a structural relationship to known skin sensitisers, whether it causes excessive skin irritation, and the nature of the dose response seen. These and other considerations are discussed in detail elsewhere (7).

DATA AND REPORTING

Data

21. Data should be summarised in tabular form showing the mean and individual DPM values and stimulation indexes for each dose (including vehicle control) group.

Test report

22. The test report should contain the following information:

Test substance:

- identification data (e.g. CAS number, if available; source; purity; known impurities; lot number);
- physical nature and physicochemical properties (e.g. volatility, stability, solubility);
- if mixture, composition and relative percentages of components.

Vehicle:

- identification data (purity; concentration, where appropriate; volume used);
- justification for choice of vehicle.

Test animals:

- strain of mice used;
- microbiological status of the animals, when known;
- number, age and sex of animals;
- source of animals, housing conditions, diet, etc.

Test conditions:

- details of test substance preparation and application;
- justification for dose selection (including results from range finding study, if conducted);-
  vehicle and test substance concentrations used, and total amount of substance applied;
- details of food and water quality (including diet type/source, water source).

Reliability check:

- a summary of results of latest reliability check, including information on substance, concentration and vehicle used;
- concurrent and/or historical positive and negative control data for testing laboratory.

Results:

- individual weights of animals at start of dosing and at scheduled kill;
- a table of mean/median (pooled approach) and individual (individual approach) DPM values, as well as the range of values for both approaches, and stimulation indices for each dose (including vehicle control) group;
- statistical analysis, where appropriate;
- time course of onset and signs of toxicity, including dermal irritation at site of administration, if any, for each animal.

Discussion of results:

- A brief commentary on the results, the dose-response analysis, and statistical analyses, where appropriate, with a conclusion as to whether the test substance should be considered a skin sensitiser.

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Appendix G5

OECD Test Guideline 406: Skin Sensitisation
(Adopted July 1992)

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406
Adopted:
17.07.92

OECD GUIDELINE FOR TESTING OF CHEMICALS

Adopted by the Council on 17th July 1992

Skin Sensitisation

INTRODUCTION

1. OECD Guidelines for Testing of Chemicals are periodically reviewed in light of scientific progress. In such reviews, special attention is given to possible improvements in relation to animal welfare. This updated version of the original guideline 406, adopted in 1981, is the outcome of a meeting of OECD experts held in Paris in May 1991.

2. Currently, quantitative structure-activity relationships and *in vitro* models are not yet sufficiently developed to play a significant role in the assessment of the skin-sensitisation potential of substances which therefore must continue to be based on *in vivo* models.

3. The guinea pig has been the animal of choice for predictive sensitisation tests for several decades. Two types of tests have been developed: adjuvant tests in which sensitisation is potentiated by the injection of Freunds Complete Adjuvant (FCA), and non-adjuvant tests. In the original guideline 406, four adjuvant tests and three non-adjuvant tests were considered to be acceptable. In this updated version, the Guinea Pig Maximisation Test (GPMT) of Magnusson and Kligman which uses adjuvant (1)(2)(3)(4) and the non-adjuvant Buehler Test (5)(6) are given preference over other methods and the procedures are presented in detail. It is recognised, however, that there may be circumstances where other methods may be used to provide the necessary information on sensitisation potential.

4. The immune system of the mouse has been investigated more extensively than that of the guinea pig. Recently, mouse models for assessing sensitisation potential have been developed that offer the advantages of an endpoint which is measured objectively, short duration and minimal animal treatment. The mouse ear swelling test (MEST) and the local lymph node assay (LLNA) appear to be promising. Both assays have undergone validation in several laboratories (7)(8)(9)(10)(11) and it has been shown that they are able to detect reliably moderate to strong sensitisers. The LLNA or the MEST can be used as a first stage in the assessment of skin sensitisation potential. If a positive result is seen in either assay, a test substance may be designated as a potential sensitiser, and it may not be necessary to conduct a further guinea pig test. However, if a negative result is seen in the LLNA or MEST, a guinea pig test (preferably a GPMT or Buehler Test) must be conducted using the procedure described in this guideline.

5. Definitions used are set out in the Annex.

GENERAL PRINCIPLE OF SENSITISATION TESTS IN GUINEA PIGS

6. The test animals are initially exposed to the test substance by intradermal injection and/or epidermal application (induction exposure). Following a rest period of 10 to 14 days (induction

period), during which an immune response may develop, the animals are exposed to a challenge dose. The extent and degree of skin reaction to the challenge exposure in the test animals is compared with that demonstrated by control animals which undergo sham treatment during induction and receive the challenge exposure.

ELEMENTS COMMON TO SENSITISATION TESTS IN GUINEA PIGS

Sex of animals

7. Male and/or female healthy young adult animals can be used. If females are used they should be nulliparous and non-pregnant.

Housing and feeding conditions

8. The temperature of the experimental animal room should be 20ºC (± 3ºC) and the relative humidity 30-70 per cent. Where the lighting is artificial, the sequence should be 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. It is essential that guinea pigs receive an adequate amount of ascorbic acid.

Preparation of the animals

9. Animals are acclimatised to the laboratory conditions for at least 5 days prior to the test. Before the test, animals are randomised and assigned to the treatment groups. Removal of hair is by clipping, shaving or possibly by chemical depilation, depending on the test method used. Care should be taken to avoid abrading the skin. The animals are weighed before the test commences and at the end of the test.

Reliability check

10. The sensitivity and reliability of the experimental technique used should be assessed every six months by use of substances which are known to have mild-to-moderate skin sensitisation properties.

11. In a properly conducted test, a response of at least 30% in an adjuvant test and at least 15% in a non-adjuvant test should be expected for mild/moderate sensitisers. Preferred substances are hexyl cinnamic aldehyde (CAS No. 101-86-0), mercaptobenzothiazole (CAS No. 149-30-4) and benzocaine (CAS No. 94-09-7). There may be circumstances where, given adequate justification, other control substances meeting the above criteria may be used.

Removal of the test substance

12. If removal of the test substance is considered necessary, this should be achieved using water or an appropriate solvent without altering the existing response or the integrity of the epidermis.

DESCRIPTION OF THE GUINEA-PIG MAXIMISATION TEST METHOD

Number of animals

13. A minimum of 10 animals is used in the treatment group and at least 5 animals in the control group. When fewer than 20 test and 10 control guinea pigs have been used, and it is not possible to conclude that the test substance is a sensitiser, testing in additional animals to give a total of at least 20 test and 10 control animals is strongly recommended.

Dose levels

14. The concentration of test substance used for each induction exposure should be well-tolerated systemically and should be the highest to cause mild-to-moderate skin irritation. The concentration used for the challenge exposure should be the highest non-irritant dose. The appropriate concentrations can be determined from a pilot study using two or three animals. Consideration should be given to the use of FCA-treated animals for this purpose.

Induction: Intradermal Injections

Day 0 - treated group

15. Three pairs of intradermal injections of 0.1 ml volume are given in the shoulder region which is cleared of hair so that one of each pair lies on each side of the midline.

Injection 1: a 1:1 mixture (v/v) FCA/water or physiological saline

Injection 2: the test substance in an appropriate vehicle at the selected concentration

Injection 3: the test substance at the selected concentration formulated in a 1:1 mixture (v/v) FCA/water or physiological saline.

16. In injection 3, water soluble substances are dissolved in the aqueous phase prior to mixing with FCA. Liposoluble or insoluble substances are suspended in FCA prior to combining with the aqueous phase. The concentration of test substance shall be equal to that used in injection 2.

17. Injections 1 and 2 are given close to each other and nearest the head, while 3 is given towards the caudal part of the test area.

Day 0 - control group

18. Three pairs of intradermal injections of 0.1 ml volume are given in the same sites as in the treated animals.

Injection 1: a 1:1 mixture (v/v) FCA/water or physiological saline

Injection 2: the undiluted vehicle

Injection 3: a 50% w/v formulation of the vehicle in a 1:1 mixture (v/v) FCA/water or physiological saline.

Induction: Topical Application

Day 5-7 - treated and control groups

19. Approximately twenty-four hours before the topical induction application, if the substance is not a skin irritant, the test area, after close-clipping and/or shaving is painted with 0.5 ml of 10% sodium lauryl sulphate in vaseline, in order to create a local irritation.

Day 6-8 - treated group

20. The test area is again cleared of hair. A filter paper (2 x 4 cm) is fully-loaded with test substance in a suitable vehicle and applied to the test area and held in contact by an occlusive dressing for 48 hours. The choice of the vehicle should be justified. Solids are finely pulverised and

incorporated in a suitable vehicle. Liquids can be applied undiluted, if appropriate.

Day 6-8 - control group

21. The test area is again cleared of hair. The vehicle only is applied in a similar manner to the test area and held in contact by an occlusive dressing for 48 hours.

Challenge: Topical Application

Day 20-22 - treated and control groups

22. The flanks of treated and control animals are cleared of hair. A patch or chamber loaded with the test substance is applied to one flank of the animals and, when relevant, a patch or chamber loaded with the vehicle only may also be applied to the other flank. The patches are held in contact by an occlusive dressing for 24 hours.

Observations - treated and control groups

23. - approximately 21 hours after removing the patch the challenge area is cleaned and closely-clipped and/or shaved or depilated if necessary;

- approximately 3 hours later (approximately 48 hours from the start of the challenge application) the skin reaction is observed and recorded according to the grades shown below;

- approximately 24 hours after this observation a second observation (72 hours) is made and once again recorded.

Blind reading of test and control animals is encouraged.

TABLE: MAGNUSSON AND KLIGMAN GRADING SCALE FOR THE EVALUATION OF CHALLENGE PATCH TEST REACTIONS

0 = no visible change

1 = discrete or patchy erythema

2 = moderate and confluent erythema

3 = intense erythema and swelling

Rechallenge

24. If it is necessary to clarify the results obtained in the first challenge, a second challenge (i.e. a rechallenge), where appropriate with a new control group, should be considered approximately one week after the first one. A rechallenge may also be performed on the original control group.

Clinical observations

25. All skin reactions and any unusual findings, including systemic reactions, resulting from induction and challenge procedures should be observed and recorded. Other procedures, e.g.

histopathological examination, the measurement of skin fold thickness, may be carried out to clarify doubtful reactions.

DESCRIPTION OF THE BUEHLER TEST METHOD

Number of animals

26. A minimum of 20 animals is used in the treatment group and at least 10 animals in the control group.

Dose levels

27. The concentration of test substance used for each induction exposure should be the highest to cause mild irritation. The concentration used for the challenge exposure should be the highest non-irritating dose. The appropriate concentration can be determined from a pilot study using two or three animals.

28. For water soluble test materials, it is appropriate to use water or a dilute non-irritating solution of surfactant as the vehicle. For other test materials 80% ethanol/water is preferred for induction and acetone for challenge.

Induction: Topical application

Day 0 - treated group

29. One flank is cleared of hair (closely-clipped). The test patch system should be fully loaded with test substance in a suitable vehicle (the choice of the vehicle should be justified; liquid test substances can be applied undiluted, if appropriate). The test patch system is applied to the test area and held in contact with the skin by an occlusive patch or chamber and a suitable dressing for 6 hours.

30. The test patch system must be occlusive. A cotton pad is appropriate and can be circular or square, but should approximate 4-6 cm^2. Restraint using an appropriate restrainer is preferred to assure occlusion. If wrapping is used, additional exposures may be required.

Day 0 - control group

31. One flank is cleared of hair (closely-clipped). The vehicle only is applied in a similar manner to that used for the treated group. The test patch system is held in contact with the skin by an occlusive patch or chamber and a suitable dressing for 6 hours. If it can be demonstrated that a sham control group is not necessary, a naive control group may be used.

Days 6-8 and 13-15 - treated and control groups

32. The same application as on day 0 is carried out on the same test area (cleared of hair if necessary) of the same flank on day 6-8, and again on day 13-15.

Challenge

Day 27-29 - treated and control groups

33. The untreated flank of treated and control animals is cleared of hair (closely-clipped). An occlusive patch or chamber containing the appropriate amount of test substance is applied, at the maximum non-irritant concentration, to the posterior untreated flank of treated and control animals.

When relevant, an occlusive patch or chamber with vehicle only is also applied to the anterior untreated flank of both treated and control animals. The patches or chambers are held in contact by a suitable dressing for 6 hours.

Observations - treated and control groups

34. - approximately 21 hours after removing the patch the challenge area is cleared of hair;

- approximately three hours later (approximately 30 hours after application of the challenge patch) the skin reactions are observed and recorded according to the grades shown in the Guinea-Pig Maximisation Test (see paragraph 23);

- approximately 24 hours after the 30 hour observation (approximately 54 hours after application of the challenge patch) skin reactions are again observed and recorded.

Blind reading of test and control animals is encouraged.

Rechallenge

35. If it is necessary to clarify the results obtained in the first challenge, a second challenge (i.e. a rechallenge), where appropriate with a new control group, should be considered approximately one week after the first one. The rechallenge may also be performed on the original control group.

Clinical observations

36. All skin reactions and any unusual findings, including systemic reactions, resulting from induction and challenge procedures should be observed and recorded. Other procedures, e.g. histopathological examination, measurement of skin fold thickness, may be carried out to clarify doubtful reactions.

DATA AND REPORTING (GPMT and Buehler Test)

Data

37. Data should be summarised in tabular form, showing for each animal the skin reactions at each observation.

Test report

38. The test report must include the following information:

Test substance:

- physical nature and, where relevant, physicochemical properties;
- identification data.

Vehicle:

- justification of choice of vehicle.

Test animals:

- strain of guinea-pig used;

- number, age and sex of animals;
- source, housing conditions, diet, etc.;
- individual weights of animals at the start and at the conclusion of the test.

Test conditions:

- technique of patch site preparation;
- details of patch materials used and patching technique;
- result of pilot study with conclusion on induction and challenge concentrations to be used in the test;
- details of test substance preparation, application and removal;
- vehicle and test substance concentrations used for induction and challenge exposures and the total amount of substance applied for induction and challenge.

Reliability check:

- a summary of the results of the latest reliability check including information on substance, concentration and vehicle used.

Results:

- on each animal including grading system;
- narrative description of the nature and degree of effects observed;
- any histopathological findings.

Discussion of the results.

If a screening assay is performed before the guinea pig test the description or reference of the test, including details of the procedure, must be given together with results obtained with the test and reference substances.

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ANNEX

DEFINITIONS

Skin sensitisation (allergic contact dermatitis) is an immunologically mediated cutaneous reaction to a substance. In the human, the responses may be characterised by pruritis, erythema, oedema, papules, vesicles, bullae or a combination of these. In other species the reactions may differ and only erythema and oedema may be seen.

Induction exposure: an experimental exposure of a subject to a test substance with the intention of inducing a hypersensitive state.

Induction period: a period of at least one week following an induction exposure during which a hypersensitive state may develop.

Challenge exposure: an experimental exposure of a previously treated subject to a test substance following an induction period, to determine if the subject reacts in a hypersensitive manner.

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National Toxicology Program
P.O. Box 12233
Research Triangle Park, NC 27709

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
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